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<b>(21) International Application Number:</b> PCT/US95/14974 <b>(22) International Filing Date:</b> 16 November 1995 (16.11.95) <b>(30) Priority Data:</b> 08/341,018 17 November 1994 (17.11.94) US <b>(71) Applicant:</b> CAMBRIDGE NEUROSCIENCE, INC. [US/US]; One Kendall Square, Building 700, Cambridge, MA 02139 (US). <b>(71)(72) Applicants and Inventors:</b> GWYNNE, David, I. [CA/US]; 77 Grover Street, Beverly, MA 01915 (US). MAHANTHAPPA, Nagesh, K. [US/US]; 240 Norfolk Street, Cambridge, MA 02139 (US). MARCHIONNI, Mark, A. [US/US]; 24 Twin Circle Drive, Arlington, MA 02174 (US). BERMINGHAM-MCDONOGH, Olivia [IE/US]; 18815 Cranbrook Avenue, Torrance, CA 90504 (US). GOLDIN, Stanley, M. [US/US]; 10 Russell Road, Lexington, MA 02173 (US). MCBURNEY, Robert, N. [AU/US]; 20 Leslie Road, Newton, MA 02166 (US). <b>(74) Agent:</b> BUTLER, Gregory, B.; Cambridge NeuroScience, Inc., Building 700, One Kendall Square, Cambridge, MA 02139 (US).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, LR, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> USE OF NEUREGULINS AS MODULATORS OF CELLULAR COMMUNICATION  <b>(57) Abstract</b>  The present invention relates to methods of affecting cellular communication in a vertebrate. The communication is affected by the administration of a neuregulin to a vertebrate, where the neuregulin interacts with a first cell type which results in the production of a product (i.e., Product A). This product, in turn, affects the function of a second cell type. Methods are disclosed in which the affect in function of the second cell type, results in the production of a second product (i.e., Product B) which, in turn, can affect the function of the first cell type or a third cell type. Additional methods are included for treatment of disorders involving an altered or inadequate level of production of a product involved in cellular communication.		

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## USE OF NEUREGULINS AS MODULATORS OF CELLULAR COMMUNICATION

### 5 FIELD OF THE INVENTION

This invention relates to methods of affecting cellular communication.

### BACKGROUND OF THE INVENTION

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Vertebrate cells depend on externally produced factors for growth, differentiation and survival. These factors can be in the form of diffusible molecules that act at a distance from their site of synthesis. Alternatively these factors can be in the form of cell-surface-bound molecules that rely on cell-to-cell contact for their  
15 function. In many cases, different cell types may interact in a reciprocal manner in that both cell types produce factors that affect the other cell type. Vertebrates rely on these reciprocal interactions during embryogenesis and during the response to injury and disease.

20

Interdependence of cells and tissues plays important roles in the vertebrate nervous system. The nervous system is composed of neurons and neuroglial support cells. Peripheral nervous system axons are ensheathed by neuroglial cells (Schwann cells) and target organs which include skin, sensory receptors, muscle and other neurons. Additionally, peripheral axons interact with components of the central  
25 nervous system in the spinal cord. These include neurons and neuroglial cells such as astrocytes and oligodendrocytes .

30

It is well established that neurons and the tissues and cells with which they interact are dependent on each other for trophic support. This relationship is mediated  
30 by factors (proteins) produced by neurons that maintain the viability of target tissues (e.g. motor neuron derived factors that maintain muscle integrity) and neurotrophic factors produced by target (and other) tissues that maintain neuronal viability (e.g. muscle derived factors that maintain motor neuron viability). This interdependence plays an important role in embryonic development, maintenance of viability and  
35 response to injury in the nervous system and its targets.

The survival of various neuronal populations has been thought to be dependent only upon neurotrophic factors produced by targets of innervation. Recently it has been realized that neurotrophic factors are also derived from axonally associated cells

(periaxonal glia), soma associated (perisomatic) cells (e.g. glia and efferent synapses) and from autocrine sources. These proteins are taken up by neurons where they exert their effect at the cell body. Neurotrophic factors either maintain the viability of the neuron or induce specific effects such as axonal extension, sprouting and other responses to injury and disease. Examples include factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and related molecules as well as ciliary neurotrophic factor (CNTF), insulin like growth factor (IGF) and fibroblast growth factors (FGF's) that all have neurotrophic activity and are derived from neuronally associated tissues as diverse as muscle, Schwann cells and spinal cord astrocytes and other neurons (e.g., Nishi, *Science* (1994) 265:1052).

The identification of pharmaceutical products or agents which induce the endogenous production of trophic factors would be beneficial treatment of diseases which involve trophic support.

## SUMMARY OF THE INVENTION

In general, the present invention relates to methods of affecting cellular communication in a vertebrate. The communication is affected by the administration of  
5 a neuregulin to a vertebrate, where the neuregulin interacts with a first cell type which results in the production of a product or products (i.e., Product(s) A). This product, in turn, affects the function of a second cell type (see Figures 9 and 10).

Neuregulins are a family of protein factors encoded by one gene. A variety of  
10 messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding and activation to erbB2 (neu) and closely related receptors erbB3 and erbB4. The invention provides methods for using all of the known products of the neuregulin gene, as well as, other not yet discovered splicing variants of the neuregulin gene.

15 Methods also are provided by the invention in which the effect in function of the second cell type, as described above, results in the production of a second product (i.e., Product B) which, in turn, can affect the function of the first cell type or a third cell type (see Figures 9 and 10).

20 Included in the invention as well, are methods for treatment when disorders involve an altered or inadequate level of production of a product involved in cellular communication.

25 Advantages of the present invention include the development of new therapeutic approaches to injury or disease based on the interdependence or communication of cells and the ability to influence or affect that communication with neuregulins. For example, a neuregulin factor that is produced by the second cell type can induce the first cell type to produce a product or products (Product(s) A) that are trophic for the second  
30 cell type. More specifically, cells and tissues that are associated with neurons may be induced to respond to a neuronally produced factor (neuregulin). This response would be in the form of the production of products (Product(s) A) that are trophic for neurons. The endogenous induction of more than one neurotrophic products by the neuregulin would be more effective than the therapeutic use of a single neurotrophic factor.  
35 Neurotrophic factors generally have restricted effects on specific neuronal subtypes (e.g. CNTF is trophic for motor neurons and NGF is trophic for sympathetic neurons as well as a subset of sensory neurons). Furthermore, the types of neurotrophic factors produced by a particular tissue are probably dependent on the target neuron type as well

as the type and stage of injury. As an example, CNTF, which is trophic for motor neurons, is released by Schwann cells in the early stages of recovery from nerve injury. This is replaced within a few days by Schwann cell and muscle derived BDNF, another motor neuron trophic factor (Curtis, et al., *Nature* (1993) **365**:253-255; and Funakoshi, et al, *J. Cell Biol.* (1993) **123**:455-465). In addition multiple neurotrophic factors function *in vivo* and may be synergistic in their effects. For example, it has been shown that multiple factors more efficiently arrest disease induced neuronal degeneration in animals than the use of a single factor (Mitsumoto et al., *Science* (1994) **265**:1107).

10

In the central nervous system, the neuregulin target, the first cell type, could be a neuron that in turn produces Product(s) A. Product A then affects other tissues (the second cell type) that produce neurotrophic products (Product(s) B) that affect the second cell type (the second cell type may be the source of the neuregulin), or perhaps a third cell type.

15

Thus, the use of the neuregulins, that are trophic for neuronally associated tissues in the manner described above would be therapeutically useful. Treatment would ensure the production of target specific combinations of products that are tailored to a particular disease state.

20

## BRIEF DESCRIPTION OF THE FIGURES

- Figure 1** is a schematic diagram showing the method used to set up the SCG (superior cervical ganglion)/culture tube experiments; (1) tubes are filled with collagen +/- GGF; (2) SCG explants are placed in tubes; (3) tubes are cultured in humidified chambers; and (4) the extruded gels are fixed and stained as a "whole mount" (Anti-S100 for Schwann cells, and anti-tubulin  $\beta 3$  for axons.).
- Figure 2** is a schematic diagram of the grid reticule inserted in the microscope ocular, which at a total magnification of 160X, allowed quantification of Schwann cell outgrowth and neurite outgrowth for the SCG/culture tube experiments.
- Figure 3A** shows the control data, that is, Schwann cell number as a function of distance from the SCG explant, for the SCG/culture tube experiments.
- Figure 3B** shows experimental data, of Schwann cell outgrowth for the SCG/culture tube experiments, at a dosage of 5 ng/ml rhGGF2.
- Figure 3C** shows experimental data, of Schwann cell outgrowth for the SCG/culture tube experiments, at a dosage of 50 ng/ml rhGGF2.
- Figure 3D** shows experimental data, of Schwann cell outgrowth for the SCG/culture tube experiments, at a dosage of 500 ng/ml rhGGF2.
- Figure 4** shows the total number of Schwann cells as a function of days *in vitro* for the SCG/culture tube experiments.
- Figure 5** shows experimental data, of neurite outgrowth, as a function of distance from the SCG explant, for the SCG/culture tube experiments performed at dosage levels of 5, 50 and 500 ng/ml rhGGF2.
- Figure 6A** shows a 2-dimensional dose-response matrix, used to examine the effects of rhGGF2 on neuronal survival and outgrowth.
- Figure 6B** illustrates the manner of counting, used in the afore-mentioned 2-dimensional dose-response experiment, by showing a representative sample well with fields of view.

**Figure 7** shows experimental data of the effects of rhGGF2 on neuronal survival and outgrowth.

**Figure 8A** shows data on the effects of exogenous GGF on the number of myelinated axons at 28 days post-injury.

**Figure 8B** shows the above-referenced data in bar graph form.

**Figure 9** represents a schematic illustration of the effect neuregulins can have on cellular communication.

**Figure 10** represents a schematic illustration of specific effects of neuregulins on cellular communication within the nervous system.

**Figure 11A** is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP1 shown in Figure 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

**Figure 11B** is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP2. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

**Figure 11C** is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern of GGF2BPP3. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA).

**Figure 12** is a diagram of representative splicing variants corresponding to bovine GGF gene products. The coding segments are represented by F, E, B, A, G, C, C/D, C/D', D, D', H, K and L. The location of the peptide sequences derived from purified protein are indicated by "o."

**Figure 13** is a listing of the DNA sequences and predicted peptide sequences of the coding segments of GGF. Line 1 is a listing of the predicted amino acid sequences of bovine GGF, line 2 is a listing of the nucleotide sequences of bovine GGF, line 3 is a listing of the nucleotide sequences of human GGF (heregulin) (nucleotide base matches are indicated with a vertical line) and line 4 is a listing of the predicted amino acid sequences of human GGF/heregulin where it differs from the predicted bovine



sequence. Coding segments E, A' and K represent only the bovine sequences. Coding segment D' represents only the human (heregulin) sequence.

5 **Figure 14** is the predicted GGF2 amino acid sequence and nucleotide sequence of BPP5. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

10 **Figure 15** is the predicted amino acid sequence and nucleotide sequence of GGF2BPP2. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

15 **Figure 16** is the predicted amino acid sequence and nucleotide sequence of GGF2BPP4. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

**Figure 17** is a list of splicing variants derived from the sequences shown in Figure 13.

20 **Figure 18** is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL1.

**Figure 19** is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL2.

25 **Figure 20** is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL3.

**Figure 21** is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL4.

30 **Figure 22** is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL5.

35 **Figure 23** is the predicted amino acid sequence, bottom, and nucleic sequence, top, of EGFL6.

**Figure 24** is the predicted amino acid sequence (middle) and nucleic sequence (top) of GGF2HBS5. The bottom (intermittent) sequence represents peptide sequences derived from GGF-II preparations.

**Figure 25** is the sequences of GGFHBS5, GGFHB1 and GGFBPP5 polypeptides.

**Figure 26** is the amino acid sequence of cDNA encoding mature hGGF2.

5

**Figure 27** depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1. The figure is the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame.

## DETAILED DESCRIPTION OF THE INVENTION

It is intended that all references cited shall be incorporated herein by reference.

### 5 General

The invention pertains to methods of affecting cellular communication in vertebrates. The communication is affected by the administration of a neuregulin to a vertebrate where the neuregulin interacts with a first cell type which results in the  
10 production of a product. This product, in turn, affects the function of a second cell type. More specifically, the invention relates to the induction of endogenous tropic factors (products) by the administration of a neuregulin.

Methods also are provided by the invention in which the affect in function of the  
15 second cell type, described above, results in the production of a second product which, in turn, can affect the function of the first cell type, the second cell type or a third cell type.

### Definition of Key Terms

20

The term administration as used herein refers to the act of delivering a substance, including but not limited to the following routes: parenteral, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol,  
25 scarification, orally, buccal, rectal or vaginal. Administration as used herein refers to a pharmaceutical preparation of a substance and the delivery of that preparation to a recipient.

The term affecting as used herein refers to the induction of a quantitative change  
30 in the response of a target cell, as a result of an interaction with a Product.

The term Alzheimer's Disease as used herein refers to a progressive central neurodegeneration involving loss of cortical and other neurons, and associated with neurofibrillary tangles and  $\beta$ -amyloid deposits.

35

The term amyotrophic lateral sclerosis (ALS) as used herein refers to a motor neuron disease characterized by a progressive degeneration of the neurons that give rise to the corticospinal tract that results in a deficit in upper and lower motor neurons.

The term astrocyte as used herein refers to a neuroglial cell of ectodermal origin and its progenitor cells. This cell has a round nucleus and a "star shaped" body and many long processes that end as vascular foot plates on the small blood vessels of the CNS and is associated with other structures. A more complete definition of the astrocyte and its progenitors can be found in the following materials: Reynolds and Weiss, *Science* (1992) **255**:1707-1710; Reynolds, Tetzlaff, and Weiss, *J. Neurosci* (1992) **12**:4565-4574; and Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

10

The term cellular communication as used herein refers to the synthesis of a substance in one cell type and the interaction of that substance with a second cell type. This process includes, but is not limited to, secretion of the substance from a cell. The substance elicits a change in the second cell type or with the first cell type. Communication can occur reciprocally or non-reciprocally with one or more cell types.

15

The term differentiation as used herein refers to a morphological and/or chemical change that results in the generation of a different cell type or state of specialization. The differentiation of cells as used herein refers to the induction of a cellular developmental program which specifies one or more components of a cell type. The therapeutic usefulness of differentiation can be seen, in increases in quantity of any component of a cell type in diseased tissue by at least 10% or more, more preferably by 50% or more, and most preferably by more than 100% relative to the equivalent tissue in a similarly treated control animal.

20

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The term disorder as used herein refers to a disturbance of function and/or structure of a living organism, resulting from an external source, a genetic predisposition, a physical or chemical trauma, or a combination of the above, including but not limited to any mammalian disease.

30

The term first cell type as used herein refers to the cell type that interacts with a neuregulin. The first cell type includes but is not limited to one or more of the following: neuron, glial cell, Schwann cell, astrocyte, oligodendrocyte, myoblast, muscle cell, satellite cell, skin cell, sensory organ cell, inflammatory cell such as macrophage, neutrophil, T-cell, eosinophil, mast cell, basophil and stromal cell such as fibroblasts or endothelial cells. Bloom and Fawcett, *A Textbook of Histology*, tenth ed. (1975), W. B. Saunders Company, Philadelphia, PA.

35

The term function as used herein refers to any activity or response of a cell. These include but are not limited to proliferation, differentiation, growth, survival, changes in the pattern of gene expression and secretion, and metabolic changes.

5       The term glial cell as used herein refers to connective and support tissues of the nervous system and includes ectodermally derived astrocytes, oligodendroglia, Schwann cells and mesodermally derived microglia and their progenitors. A more complete definition of glial cells and their progenitors can be found in the following materials: Anderson, *FASEB J.* (1994) 8:707-713; Reynolds and Weiss, *Science* (1992)  
10    255:1707-1710; Reynolds, Tetzlaff, and Weiss, *J. Neurosci* (1992) 12:4565-4574; and Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

15       The term interacts as used herein refers to a contact with a target (cell), including but not limited to binding of a product to a cell receptor.

      The term mammal as used herein describes a member of the Class Mammalia (Subphylum Vertebrata).

20       The term matrix molecule as used herein refers to a chemical component of the insoluble meshwork of extracellular proteins that mediate adhesive interactions between cells and modulate the functions of cells.

25       The term mitosis as used herein refers to the division of a cell where each daughter nucleus receives identical complements of the numbers of chromosomes characteristic of the somatic cells of the species. Mitosis as used herein refers to any cell division which results in the production of new cells in the patient. More specifically, a useful therapeutic is defined *in vitro* as an increase in mitotic index relative to untreated cells of 50%, more preferably 100%, and most preferably 300%,  
30    when the cells are exposed to labeling agent for a time equivalent to two doubling times. The mitotic index is the fraction of cells in the culture which have labeled nuclei when grown in the presence of a tracer which only incorporates during S phase (i.e., BrdU) and the doubling time is defined as the average time required for the number of cells in the culture to increase by a factor of two.

35

      For example, one effect on mitosis *in vivo* is defined as an increase in satellite cell activation as measured by the appearance of labeled satellite cells in the muscle tissue of a mammal exposed to a tracer which only incorporates during S phase (i.e.,

BrdU). A useful therapeutic is defined *in vivo* as a compound which increases satellite cell activation relative to a control mammal by at least 10%, more preferably by at least 50%, and most preferably by more than 200% when the mammal is exposed to labeling agent for a period of greater than 15 minutes and tissues are assayed between 10 hours  
5 and 24 hours after administration of the mitogen at the therapeutic dose.

The term muscle cell as used herein refers to a cellular component of skeletal, smooth or cardiac muscle, including but not limited to myofibrils, satellite cells, and myoepithelial cells and their progenitors. A more complete definition of muscle cells  
10 can be found in, Wheater, et al., *Functional Histology* (1987), Churchill Livingstone, New York, NY; and *Myology*, ed. by Engel and Franzini-Armstrong, second ed. (1994) McGraw Hill, New York, NY.

The term neuregulin as used herein refers to the glial growth factors, the heregulins, neu differentiation factor, acetylcholine receptor inducing activity, and erbB2, 3 and 4 binding proteins. A more complete definition of neuregulins can be found in the specification herein and in the following materials: U.S. Patent No. 5,237,056; U.S. Patent Application SN 08/249,322; WO 92/20798; EPO 0 505 148 A1; Marchionni, et al., *Nature* **362**:313, 1993; Benveniste, et al., *PNAS* **82**:3930-3934, 1985;  
15 Kimura, et al., *Nature* (1990) **348**:257-260; Davis and Stroobant, *J. Cell. Biol.* (1990) **110**:1353-1360; Wen, et al., *Cell* (1992) **69**:559; Yarden and Ullrich, *Ann. Rev. Biochem.* (1988) **57**:443, ; Holmes, et al., *Science* **256**:1205, 1992; Dobashi, et al., *Proc. Nat'l. Acad. Sci.* **88**:8582, 1991; Lupu, et al., *Proc. Nat'l. Acad. Sci.* (1992) **89**:2287; Peles and Yarden, *BioEssays* (1993) **15**:815, Mudge, *Current Biology* (1993) **3**:361, all  
20 hereby incorporated by reference.

The term neuregulin producing cell as used herein refers to a cell that produces a neuregulin. The term refers to all producer cells including cells that produce recombinant neuregulins.  
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The term neurological disorder as described herein refers to a disorder of the nervous system.

The term nervous system cell as used herein includes nervous system support  
35 cells and neurons.

The term neuron as used herein refers to a complete nerve cell, including the cell body and all of its processes, and its progenitors. A more complete definition of neuron

and its progenitors can be found in the following materials: Reynolds and Weiss, *Science* (1992) 255:1707-1710; Reynolds, Tetzlaff, and Weiss, *J. Neurosci* (1992) 12:4565-4574; Ray, Peterson, Schinstine, and Gage, *PNAS* (1993) 90:3602-3606; and Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term neurotrophic agent as used herein refers to a substance that elicits a trophic effect in one or more neuronal subtypes. These effects include but are not limited to survival, sprouting and differentiation.

The term oligodendrocyte as used herein refers to the neuroglial cells, of ectodermal origin, with small oval nuclei and fine cytoplasmic processes that are responsible for the formation of myelin in the CNS. The progenitors of oligodendrocytes are also included. A more complete definition of oligodendrocytes and their progenitors can be found in Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term Parkinson's Disease as used herein refers to a progressive central neurodegeneration involving dopaminergic neurons.

The term peripheral neuropathy as used herein refers to functional disturbances and/or pathological changes in the peripheral nervous system.

The term Product as used herein refers to any substance as defined herein as Product A or Product B.

The term Product A as used herein refers to the substances whose synthesis and release are induced in the first cell type by neuregulin. Such substances include but are not limited to one or more of the following: nerve growth factor (NGF), neurotrophins, brain-derived neurotrophic factor, ciliary neurotrophic factor, leukemia inhibiting factor, interleukin 6, platelet derived growth factor, fibroblast growth factors, transforming growth factor  $\beta$ , epidermal growth factor, transforming growth factor  $\alpha$ , neuregulins, insulin like growth factor, matrix molecules, adhesion molecules, growth factor receptors, low affinity NGF receptor, proteases, protease inhibitors, and antioxidants.

The term Product B as used herein refers to the substances whose synthesis and release are induced in the second cell type by Product A. Such substances include but

are not limited to one or more of the following: nerve growth factor (NGF), neurotrophins, brain-derived neurotrophic factor, ciliary neurotrophic factor, leukemia inhibiting factor, interleukin 6, platelet derived growth factor, fibroblast growth factors, transforming growth factor  $\beta$ , epidermal growth factor, transforming growth factor  $\alpha$ ,  
5 neuregulins, glial derived neurotrophic factor, insulin like growth factor, matrix molecules, adhesion molecules, growth factor receptors, low affinity NGF receptor (p75), proteases, protease inhibitors and antioxidants.

The term production as used herein refers to induced or constitutive synthesis  
10 and/or release of a Product from a cell.

The term protease as used herein refers to an enzyme that hydrolyses peptide bonds in a protein molecule.

15 The term protease inhibitor as used herein refers to a molecule that inhibits the activity and/or function of a protease.

The term Schwann cell as used herein refers to the neuroglial cell composing the neurolemma of peripheral nerve fibers and its progenitors. A more complete definition  
20 of Schwann cells and their progenitors can be found in the following materials: Anderson, *FASEB J.* (1994) 8:707-713; Kandel, et al., *Principles of Neuroscience*, third ed. (1991), Appleton & Lange, Norwalk, CT.

The term second cell type as used herein refers to the cell type that interacts with  
25 and responds to Product A. The second cell type includes but is not limited to one or more of the following: neuron, glial cell, Schwann cell, astrocyte, oligodendrocyte, myoblast, muscle cell, satellite cell, skin cell, sensory organ cell, inflammatory cell such as macrophage, neutrophil, T-cell, eosinophil, mast cell, basophil and stromal cell such as fibroblasts or endothelial cells. A more complete definition may be found in Bloom  
30 and Fawcett, *A Textbook of Histology*, tenth ed. (1975), W. B. Saunders Company, Philadelphia, PA.

The term sensory organ cell as used herein refers to a primary sensory cell  
35 contained within a sensory organ and its progenitors and includes but is not limited to one or more of the following: taste cells, olfactory epithelial cell, rod and cone photoreceptors, Meisner corpuscle, Ruffini corpuscle, Merckel receptor, Pacinian corpuscle, muscle spindle cell, cochleovestibular hair cells and joint mechanoreceptor cells. A more complete definition of sensory organ cells and their progenitors can be



found in, Wheater, et al., *Functional Histology* (1987), Churchill Livingstone, New York, NY; Mahanthappa and Schwarting, *Neuron* (1993) 10:293-305; Forge, Li, Corwin and Nevill, *Science* (1993) 259:1616-1622; Tsue, Watling, Weisleder, Coltrera and Rubel, *J. Neurosci* (1994) 14:140-152.

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The term skin cell as used herein refers to the cellular components of the skin and includes fibroblasts, keratinocytes, epidermal cells, hair follicle cells, melanocytes, myoepithelial sweat gland cells, and sebaceous gland cells and their progenitors. A more complete definition of skin cells and their progenitors can be found in, Wheater, et al., *Functional Histology* (1987), Churchill Livingstone, New York, NY.

10

The term spinal muscular atrophy as used herein refers to a progressive disease of upper and lower motor neurons, usually present in childhood.

15

The term survival as used herein refers to any process where a cell avoids death. The term survival as used herein also refers to the prevention of cell loss as evidenced by necrosis or apoptosis or the prevention of other mechanisms of cell loss. Survival as used herein indicates a decrease in the rate of cell death of at least 10%, more preferably by at least 50%, and most preferably by the least 300% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture when the cells are 8 days post-differentiation (i.e., 8 days after the media is changed from 20% to 0.5% serum).

20

The term therapeutically effective amount as used herein refers to that amount which will produce a desirable result upon administration and which will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

25

The term third cell type as used herein refers to a cell type that interacts with and responds to Product B. The third cell type may be identical to the first cell type. The third cell type includes but is not limited to one or more of the following: neuron, glial cell, Schwann cell, astrocyte, oligodendrocyte, myoblast, muscle cell, satellite cell, skin cell, sensory organ cell, inflammatory cell such as macrophage, neutrophil, T-cell, eosinophil, mast cell, basophil and stromal cell such as fibroblasts or endothelial cells. A more complete definition may be found in Bloom and Fawcett, *A Textbook of Histology*, tenth ed. (1975), W. B. Saunders Company, Philadelphia, PA.

30

35

The term treating as used herein may refer to a procedure (e.g. medical procedure) designed to exert a beneficial effect on a disorder. Treating as used herein means any administration of a substance described herein for the purpose of increasing cellular communication of products. Most preferably, the treating is for the purpose of  
5 reducing or diminishing the symptoms or progression of a disease or disorder of cells. Treating as used herein also means the administration of a substance to increase or alter the cells in healthy individuals. The treating may be brought about by the contacting of the cells which are sensitive or responsive to the neuregulins described herein with an effective amount of the neuregulin.

10

The term trophic as used herein refers to an effect of a substance on a cell, including but not limited to proliferation, growth, sprouting, differentiation or survival.

The term vertebrate as used herein refers to an animal that is a member of the  
15 Subphylum Vertebrata (Phylum Chordata).

## Neuregulins

A novel aspect of the present invention relates to the ability of neuregulins to affect cellular communication between different and similar cell types. Neuregulins are the products of a gene which produce a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins. These proteins are of different lengths and contain some common peptide sequences and some unique peptide sequences. The conclusion that these factors are encoded by a single gene is supported by the differentially-spliced RNA sequences which are recoverable from bovine posterior pituitary, human spinal chord and human breast cancer cells (MDA-MB-231). Further support for this conclusion derives from the size range of proteins which act as ligands for the p185erbB2 receptor (see below).

Further evidence to support the fact that the genes encoding GGF/p185erbB2 binding proteins are homologous comes from nucleotide sequence comparison. Holmes et al., (*Science* (1992) **256**:1205-1210) demonstrate the purification of a 45-kilodalton human protein (Heregulin- $\alpha$ ) which specifically interacts with the receptor protein p185erbB2. Peles et al., (*Cell* (1992) **69**:559) describe a complementary DNA isolated from rat cells encoding a protein call "neu differentiation factor" (NDF). The translation product of the NDF cDNA has p185erbB binding activity. Several other groups have reported the purification of proteins of various molecular weights with p185erbB2 binding activity. These groups include the following: Lupu et al., (1992) *Proc. Nat'l. Acad. Sci. USA* **89**:2287; Yarden and Peles, (1991) *Biochemistry* **30**:3543; Lupu et al., (1990) *Science* **249**:1552; Dobashi et al., (1991) *Biochem. Biophys. Res. Comm.* **179**:1536; and Huang et al., (1992) *J. Biol. Chem.* **257**:11508-11512.

We have found that p185erbB2 and related receptor binding proteins (i.e., p185erbB3 and p185erbB4) affect cellular communication. This effect results in the production of a product from a first cell type, where the product, in turn affects the function of a second cell type. The affect in a function of the second cell type and can result in the production of other products which also can affect functions of other cell types. For example, neuregulins can interact with Schwann cells, which as a result of this interaction produce neurotrophic agents. These agents, in turn, interact with neurons to promote their neuronal regeneration. Alternatively, in the central nervous system, a first cell type, being a neuron, could produce a neuregulin, which in turn, affects a second cell type which is a neuron also.

These neuregulins may be identified using the protocols described herein (Examples 1 and 2) and in Holmes et al., *Science* (1992) **256**: 1205; Peles et al., *Cell* (1992) **69**:205; Wen et al., *Cell* (1992) **69**:559; Lupu et al., *Proc. Nat'l. Acad. Sci. USA* (1992) **89**:2287; Yarden and Peles, *Biochemistry* (1991) **30**:3543; Lupu et al., *Science* (1990) **249**:1552; Dobashi et al., *Biochem. Biophys. Res. Comm.* (1991) **179**:1536; Huang et al., *J. Biol. Chem.* (1992) **257**:11508-11512; Marchionni et al., *Nature* (1993) **362**:313; and in U.S. Patent Application Serial No. 07/931,041, filed August 17, 1992, all of which are incorporated herein by reference.

Specifically, the invention provides for use of polypeptides of a specified formula, and DNA sequences encoding those polypeptides. The polypeptides have the formula

WYBAZCX

wherein WYBAZCX is composed of the amino acid sequences shown in Figure 13; wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprises the polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL; provided that, either

- a) at least one of F, Y, B, A, Z, C, or X is of bovine origin; or
- b) Y comprises the polypeptide segment E; or
- c) X comprises the polypeptide segments C/D HKL, C/D D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, C/D C/D' D' HKL, C/D'H, C/D C/D'H, or C/D C/D' HL.

In addition, the invention includes the use of the DNA sequence comprising coding segments 5'FBA3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments 5'FBA3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the coding segments 5'FEBA'3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

5 the DNA sequence comprising the coding segments 5'FEBA'3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 13;

the DNA sequence comprising the polypeptide coding segments of the  
10 GGF2HBS5 cDNA clone (ATCC Deposit No. 75298, deposited September 2, 1992), also known as GGF-II.

The invention further includes the use of peptides of the formula FBA, FEBA, FBA' FEBA' and DNA sequences encoding these peptides wherein the polypeptide  
15 segments correspond to amino acid sequences shown in Figure 13. The purified GGF-II polypeptide is also included as part of the invention.

Also included in this invention is the mature GGF peptide and the DNA encoding said peptide, exclusive of the N-terminal signal sequence, which is also useful  
20 for treatment of conditions involving abnormalities in cellular communication.

Furthermore, the invention includes a method of cellular communication by the application to a vertebrate of a

- 30 kD polypeptide factor isolated from the MDA - MB 231 human breast cell  
25 line; or

- 35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or

-75 kD polypeptide factor isolated from the SKBR-3 human breast cell line; or

-44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell  
30 line, or

-25kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

-45 kD polypeptide factor isolated from the MDA - MB 231 human breast cell;

or

35 -7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or

-25 kD polypeptide factor isolated from the bovine kidney cell; or

-42 kD polypeptide factor (ARIA) isolated from brains.

The invention further includes a method for the use of the EGFL1, EGFL2, EGFL3, EGFL4, EGFL5 and EGFL6 polypeptides, Figure 18 to Figure 23 respectively, and for the methods of affecting cellular communication *in vivo* and *in vitro*.

5

Also included in the invention is the administration of the GGF-II polypeptide whose sequence is shown in Figure 24, for affecting cellular communication.

10 An additional aspect of the invention includes the use of the above-referenced peptides for the purpose of stimulating Schwann cells to produce growth factors which may, in turn, be harvested for scientific or therapeutic use.

Thus, the invention further embraces a polypeptide factor capable of affecting cellular communication and including an amino acid sequence encoded by:

- 15 (a) a DNA sequence shown in Figure 11;  
(b) a DNA sequence shown in Figure 27;  
(c) the DNA sequence represented by nucleotides 281-557 of the sequences shown in Figure 11; or  
(d) a DNA sequence hybridizable to any one of the DNA sequences  
20 according to (a), (b) or (c).

The invention further includes sequences which have greater than 60%, preferably 80%, sequence identity of homology to the sequences indicated above.

25 While the present invention is not limited to a particular set of hybridization conditions, the following protocol gives general guidance which may, if desired, be followed:

DNA probes may be labeled to high specific activity (approximately  $10^8$  to  $10^9$   
30  $^{32}\text{P}$  dmp/ $\mu\text{g}$ ) by nick-translation or by PCR reactions according to Schowalter and Sommer (*Anal. Biochem.* (1989) 177:90-94) and purified by desalting on G-150 Sephadex columns. Probes may be denatured (10 minutes in boiling water followed by immersion into ice water), then added to hybridization solutions of 80% buffer B (2g polyvinylpyrrolidone, 2g Ficoll-400, 2g bovine serum albumin, 50ml 1M Tris HCl (pH  
35 7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl sulfate, 950 ml  $\text{H}_2\text{O}$ ) containing 10% dextran sulfate at  $10^6$  dpm  $^{32}\text{P}$  per ml and incubated overnight (approximately 16 hours) at  $60^\circ\text{C}$ . The filters may then be washed at  $60^\circ\text{C}$  first in

buffer B for 15 minutes followed by three 20-minute washes in 2X SSC, 0.1% SDS then one for 20 minutes in 1XSSC, 0.1% SDS.

In other respects, the invention provides:

- 5 (a) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, whether in reducing conditions or not, of from about 30kD to about 36kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

	Lysozyme (hen egg white)	14,400
10	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
	Phosphorylase B (rabbit muscle)	97,400;

- 15 which factor has glial cell mitogenic activity including stimulating the division of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of said activity after 10 weeks incubation in 0.1 % trifluoroacetic acid at 4° C; and

- 20 (b) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, under non-reducing conditions, of from about 55 kD to about 63 kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

	Lysozyme (hen egg white)	14,400
25	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
	Phosphorylase B (rabbit muscle)	97,400;

- 30 which factor the human equivalent of which is encoded by DNA clone GGF2HBS5 described herein and is capable of affecting cellular communication.

- 35 For convenience of description only, the lower molecular weight and higher molecular weight factors of this invention are referred to hereafter as "GGF-I" and "GGF-II", respectively. The "GGF2" designation is used for all clones isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3).

It will be appreciated that the molecular weight range limits quoted are not exact, but are subject to slight variations depending upon the source of the particular polypeptide factor. A variation of, say, about 10% would not, for example, be impossible for material from another source.

5

Another important aspect of the invention is a DNA sequence encoding a polypeptide capable of affecting cellular communication and comprising:

- (a) a DNA sequence shown Figure 11;
- (b) a DNA sequence shown in Figure 27;
- 10 (c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 11; or
- (d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

15

Thus other important aspects of the invention are:

(a) A series of human and bovine polypeptide factors capable of affecting cellular communication. These peptide sequences are shown in Figures 13, 14, 15 and 16, respectively.

20 (b) A series of polypeptide factors capable of affecting cellular communication and purified and characterized according to the procedures outlined by Lupu et al., *Science* (1990) **249**:1552; Lupu et al., *Proc. Nat'l. Acad. Sci USA* (1992) **89**:2287; Holmes et al., *Science* (1992) **256**:1205; Peles et al., *Cell* (1992) **69**:205; Yarden and Peles, *Biochemistry* (1991) **30**:3543; Dobashi et al., *Proc. Nat'l. Acad. Sci.* (1991) **88**: 8582; Davis et al., *Biochem. Biophys. Res. Commun.* (1991) **179**:1536 ; Beaumont et al., Patent Application PCT/US91/03443 (1990); Greene et al., Patent Application PCT/US91/02331 (1990); Usdin and Fischbach, *J. Cell. Biol.* (1986) **103**:493-507; Falls et al., *Cold Spring Harbor Symp. Quant. Biol.* (1990) **55**:397-406; Harris et al., *Proc. Nat'l. Acad. Sci. USA* (1991) **88**:7664-7668; and Falls et al., *Cell* (1993) **72**:801-815.

25

30 (c) A polypeptide factor (GGFBPP5) capable of affecting cellular communication. The amino acid sequence is shown in Figure 14, and is encoded by the bovine DNA sequence shown in Figure 14.

35 The novel human peptide sequences described above and presented in Figures 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.



Other compounds in particular, peptides, which bind specifically to the p185<sup>erbB2</sup> receptor and related receptors can also be used according to the invention as affections of cellular communication. A candidate compound can be routinely screened for p185<sup>erbB2</sup> binding, and, if it binds, it can then be screened for affecting cellular communication using the methods described herein.

The invention includes any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. By way of illustration, in EP-A 109748, mutations of native proteins are disclosed in which the possibility of unwanted disulfide bonding is avoided by replacing any cysteine in the native sequence which is not necessary for biological activity with a neutral amino acid. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The new sequences of the invention open up the benefits of recombinant technology. The invention thus also includes the following aspects:

(a) DNA constructs comprising DNA sequences as defined above in operable reading frame position within vectors (positioned relative to control sequences so as to permit expression of the sequences) in chosen host cells after transformation thereof by the constructs (preferably the control sequence includes regulatable promoters, e.g. Trp). It will be appreciated that the selection of a promoter and regulatory sequences (if any) are matters of choice for those of skill in the art:

(b) host cells modified by incorporating constructs as defined in (a) immediately above so that said DNA sequences may be expressed in said host cells - the choice of host is not critical, and chosen cells may be prokaryotic or eukaryotic and may be genetically modified to incorporate said constructs by methods known in the art; and,

(c) a process for the preparation of factors as defined above comprising cultivating the modified host cells under conditions permitting expression of the DNA sequences. These conditions can be readily determined, for any particular embodiment, by those of skill in the art of recombinant DNA technology. Glial cell mitogens prepared by this means are included in the present invention.

None of the factors described in the art has the combination of characteristics possessed by the present new polypeptide factors.

The invention also includes a neuregulin as defined above, by extracting vertebrate brain material to obtain protein, subjecting the resulting extract to chromatographic purification by hydroxyapatite HPLC and then subjecting these fractions to SDS-polyacrylamide gel electrophoresis. The fraction which as an  
5 observed molecular weight of about 30kD to 36kD and/or the fraction which has an observed molecular weight of about 55kD to 63kD is collected. In either case, the fraction is subjected to SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

	Lysozyme (hen egg white)	14,400
10	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
15	Phosphorylase B (rabbit muscle)	97,400

In the case of the smaller molecular weight fraction, the SDS-polyacrylamide gel is run in non-reducing conditions or in reducing conditions and in the case of the larger molecular weight fraction, the gel is run under non-reducing conditions. The fractions are then tested for activity stimulating the division of rat Schwann cells against  
20 a background of fetal calf plasma.

Preferably, the above process starts by isolating a relevant fraction obtained by carboxymethyl cellulose chromatography, e.g. from bovine pituitary material. It is also preferred that hydroxyapatite HPLC, cation exchange chromatography, gel filtration,  
25 and/or reversed-phase HPLC be employed prior to the SDS-Polyacrylamide gel electrophoresis. At each stage in the process, activity may be determined using Schwann cell incorporation of radioactive iododeoxyuridine as a measure in an assay generally as described by Brockes in *Meth. Enz.* (1987) **147**:217-225, but modified by substituting 10% FCP for 10% FCS. As already noted, such an assay is an aspect of the  
30 invention in its own substance for CNS or PNS cell, e.g. Schwann cell, mitogenic effects.

Compounds may be assayed for their usefulness *in vitro* using the methods provided in the description and examples below. Following the *in vitro* demonstration  
35 of the effect of neuregulins on cellular communication between various cell types, the *in vivo* therapeutic benefit of the effect can be accomplished by the administration of neuregulins, neuregulin producing cells or DNA encoding neuregulins to a vertebrate

requiring therapy. In a specific example, *in vivo* testing can be demonstrated as described in Example 3.

5 The invention includes the use of the above named family of proteins (i.e. neuregulins) as extracted from natural sources (tissues or cell lines) or as prepared by recombinant means.

10 Other compounds in particular, peptides, which bind specifically to the p185erbB2 and related receptor binding proteins (i.e., p185erbB3 and p185erbB4) can also be used according to the invention as effectors of cellular communication. A candidate compound can be routinely screened for p185erbB2, p185erbB3 and p185erbB4 binding, and if it binds, can then be screened for affecting cellular communication using the methods described herein.

15 The invention includes use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity related to affecting cellular communication. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be  
20 construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The human peptide sequences described above represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from  
25 natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

30 The invention includes methods for the use of any protein which is substantially homologous to the coding segments in Figure 13, as well as other naturally occurring GGF polypeptides for the purpose of inducing muscle mitogenesis. Also included are the use of: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see *Current Protocols in*  
35 *Molecular Biology*, (1989) John Wiley & Sons, New York, NY, 6.3.1 - 6.3.6, hereby incorporated by reference); and the use of polypeptides or proteins specifically bound by antisera to GGF polypeptides. The term also includes the use of chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 13.

### Use of Neuregulins

A novel aspect of the invention involves the use of neuregulins as factors to promote cell communication by inducing the production of products. These Products  
5 affect the function of these cells. Treatment of the cells to achieve these effects may be achieved by contacting cells with a polypeptide described herein.

The methods of the invention may also be used to treat a patient suffering from a disease caused by a lack of trophic factor(s). The lack of trophic factor(s) is defined by  
10 a decreased amount of trophic factor(s) relative to that of an unaffected individual sufficient to cause detectable alteration in the biological effect of those trophic factor(s). The neurotrophic factor(s) may be present at levels 10% below those observed in unaffected individuals. More preferably, the factor(s) are present at levels 20% lower than that observed in unaffected individuals, and most preferably the levels are lowered  
15 by 80% relative to unaffected individuals under similar circumstances.

The methods of the invention make use of the fact that the neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding  
20 to p185<sup>erbB2</sup> and related receptors and activation of the same. This invention provides a use for all of the known products of the neuregulin gene (described herein and in the references listed above). Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

The invention also relates to the use of other, not yet naturally isolated, splicing variants of the neuregulin gene. Figure 12 shows the known patterns of splicing. These patterns are derived from polymerase chain reaction experiments (on reverse transcribed RNA), analysis of cDNA clones (as presented within), and analysis of published sequences encoding neuregulins (Peles et al., *Cell* (1992) 69:205 and Wen et al., *Cell*  
30 (1992) 69:559). These patterns, as well as additional patterns disclosed herein, represent probable splicing variants which exist. The splicing variants are fully described in Goodearl et al., USSN 08/036,555, filed March 24, 1993, incorporated herein by reference.

35 More specifically, effects on cell communication may be achieved by contacting cells with a polypeptide defined by the formula

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 13; wherein W comprises the polypeptide segment F, or is absent wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprises the polypeptide segment C/D HKL, C/D H, C/D  
 5 HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.

Furthermore, the invention includes a method of treating muscle cells by the  
 10 application to the muscle cell of a  
     -30kD polypeptide factor isolated from the MDA-MB 231 human breast cell line; or  
     -35kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell; or  
 15      -75kD polypeptide factor isolated from SKBR-3 human breast cell line; or  
     -44kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line; or  
     -25kD polypeptide factor isolated from activated mouse peritoneal macrophages; or  
 20      -45kD polypeptide factor isolated from the MDA-MB 231 human breast cell; or  
     -7 to 14kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or  
     -25kD polypeptide factor isolated from the bovine kidney cells; or  
     -42kD ARIA polypeptide factor isolated from brain; or  
 25      -46-47kD polypeptide factor which stimulates 0-2A glial progenitor cells; or  
     -43-45kD polypeptide factor, GGFIII, U.S. patent application Serial No. 07/931,041, filed August 17, 1992, incorporated herein by reference.

The invention includes use of any modifications or equivalents of the above  
 30 polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

35

The human peptide sequences described above and presented in Figures 13, 14, 15, and 16, respectively, represent a series of splicing variants which can be isolated as full-length complementary DNAs (cDNAs) from natural sources (cDNA libraries

prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

5 Another aspect of the invention is the use of a pharmaceutical or veterinary formulation comprising any factor as defined above formulated for pharmaceutical or veterinary use, respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

10

A medicament is made by administering the polypeptide with a pharmaceutically effective carrier.

15 Thus, the formulations to be used as a part of the invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, transdermal and by other slow release devices (i.e., osmotic pump-driven devices; see also U.S.S.N. 08/293,465, hereby incorporated by reference) and also oral, buccal, rectal or vaginal  
20 administration.

The formulations of this invention may also be administered by the transplantation into the patient of host cells expressing the DNA encoding polypeptides which are effective for the methods of the invention or by the use of surgical implants  
25 which release the formulations of the invention.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

30

Methods well-known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or  
35 hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable

infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

The present factors can be used as the sole active agents, or can be used in combination with other active ingredients, e.g., other growth factors which could facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1  $\mu$ g/kg to about 1 g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a disease or disorder. The "GGF2" designation is used for all clones which were previously isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3) and, when present alone (i.e., GGF2 or rhGGF2), to indicate recombinant human protein encoded by plasmids isolated with peptide sequence data derived from the GGF-II protein (i.e., as produced in insect cells from the plasmid HBS5). Recombinant human GGF from the GGFHBS5 clone is called GGF2, rhGGF2 and GGF2HBS5 polypeptide.

Methods for treatment of diseases or disorders using neuregulins in this manner are also part of the invention. Administration of neuregulins to induce the production of a substance or substances from a neuregulin responsive cell can be used in any disorder where an increase in a neuregulin inducible substance that is trophic for the disease

- affected neurons would be of benefit. In peripheral nerve injury or peripheral nerve disorders such as the neuropathies administration of neuregulins will elicit the production of neurotrophic substances from known neuregulin target tissues such as Schwann cells and muscle. These induced substances can enhance axonal repair.
- 5 Alzheimer's disease is another target for neuregulin therapy. In the brain, neuregulins are detectable in cholinergic motor neurons (Chen, et al., *J. Comparative Neurology* (1994) **349**:389-400), these neurons degenerate in Alzheimer's disease and many show trophic responses to neurotrophic factors such as NGF. Neuregulins can be used to induce the synthesis of neurotrophic factors in those neurons that interact with
- 10 cholinergic neurons. Similar therapeutic approaches may be used in other neurodegenerative disorders such as Parkinson's disease, amyotrophic lateral sclerosis, spinal muscular atrophy or any disease where stimulation of the synthesis of substances that are trophic for disease affected neurons might be of benefit.
- 15 Methods for treatment of diseases or disorders using nucleic acid constructs encoding neuregulins or neuregulin producer cells are also part of the invention.
- 20 Delivery of DNA to a cell or tissue that will take up the DNA, express the DNA and produce neuregulin as shown by Wolff et al., (*Science* (1990) **247**:1465) and Ascadi et al., (*Nature* (1991) **352**:815) is an aspect of the invention. The neuregulin produced by this method will act on the first cell type and elicit the responses described above. Genetic modification of cultured cells (or their precursors) such as fibroblasts (as shown by Wolff et al. *Proc. Nat'l Acad. Sci. USA* (1988) **86**:1575 ) or such as those derived from the nervous system (as shown by Weiss et al. International Patent Application
- 25 number PCT/US94/01053; publication number WO 94/16718) to induce the production of neuregulin from the cultured cells is another aspect of this invention. The genetically modified neuregulin producer cells can be transplanted to a position near the first cell type and elicit the responses described above.



### Assays for Determining Neuregulin Effect(s) on Cellular Communication

Described below are generic methods for detecting the ability of a neuregulin to induce in a first cell type, the production of a product (Product A) that is trophic for a second cell type. A general reference on cell and tissue culture is *Cell and Tissue Culture: Laboratory Procedures* (Ed. by A. Doyle, J. B. Griffiths, and D. G. Newell, John Wiley and Sons, New York, NY, 1994). General references on the culture of neural cells and tissues are *Methods in Neurosciences, Vol. 2* (Ed. by P. M. Conn. Academic Press, Sand Diego, CA, 1990) and *Culturing Nerve Cells* (Ed. by G. Banker and K. Goslin, MIT Press, Cambridge, MA 1991). General references of immunocytochemistry are *Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), and *Immunocytochemistry II* (Ed. by A. C. Cuellar, John Wiley and Sons, New York, NY, 1993).

15

The vertebrate cells used in this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.* (1979) 58:44; Barnes and Sato, *Anal. Biochem.* (1980) 102:255; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195 and U.S. Pat. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression and will be apparent to the ordinarily skilled artisan.

30

**Method I -**

The use of separate cultures of a first cell type and a second cell type, to demonstrate that neuregulin induces the first cell type to produce a secreted substance that is trophic  
5 for the second cell type.

1. Establish cultures of cells from the tissue of interest (e.g. spinal cord, pancreas, gut, etc.). These cultures are enriched for the first cell type such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of  
10 immunocytochemical and/or enzymatic markers (e.g. tubulin  $\beta$ 3 for neurons (A. Banerjee, M. C. Roach, P. Trcka, and R. F. Luduena, Increased microtubule assembly in bovine brain tubulin lacking the type III isotype of  $\beta$  tubulin. *J. Biol. Chem.* (1990) **265**:1794-1799), Islet-1 for pancreatic islet cells (O. Karlsson, S. Thor, T. Norbert, H. Ohlsson, and T. Edlund, Insulin gene enhanced binding protein Isl-1 is a member of a  
15 novel class of proteins containing both a homeo and a Cys-His domain. *Nature* (1990) **344**:879-882)).
2. Establish cultures of cells from the same tissue of interest as in step 1. These cultures are enriched for the second cell type such that preferably greater than 90% of  
20 the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers.
3. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 minute and less than 7 days. At the end of the  
25 culture period, collect the conditioned culture medium, remove debris by centrifugation (200 g, 10 minutes) and filtration (nylon filter, 0.22 mm pore size). This medium (conditioned medium) will contain the secreted product(s) of the first cell type, Product A.
- 30 4. Replace or supplement the medium of the second cell type cultures with media prepared as in step 3. Include among these medium samples, medium that has been conditioned by the first cell type cultures in the absence of neuregulin (control conditioned medium). Include among the medium samples, media containing neuregulin that have not been conditioned by the first cell type cultures (non-  
35 conditioned medium).
5. Maintain the second cell type cultures as described in step 4 for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of

cellular phenotype such as, but not limited to, cell survival, morphology, production of enzymes and secreted products, etc.

5 **6. Assess the effects of the neuregulin. The neuregulin is trophic for the first cell type in a manner that promotes the production of products trophic for the second cell type if:**

a. Medium conditioned by the first cell type cultures in the presence of neuregulin maintains or increases desired aspects of cellular phenotype such as, but not limited to cell survival, morphology, production of enzymes and secreted products, etc.;

10      b.      equal volumes of control conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.);

and

c. equal volumes of non-conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

The induction by neuregulin of a secreted product, Product A, such that Product A affects a third cell type, can also be tested as in Method I. Establish cultures of cells from the same tissue of interest as in step 1. These cultures are enriched for the third cell type, such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers. Substitute the third cell type cultures for the second cell type cultures in steps 4-6.

25 If Product A is not secreted, but is bound to the surface of the first cell type, or is bound to insoluble extracellular matrix associated with the first cell type, an alternative procedure is to be used:

**Method II -**

The use of separate cultures of the first and second cell types, to demonstrate that neuregulin induces the first cell type to produce a substance on its surface that is trophic for the second cell type.

1. Establish cultures of cells from the tissue of interest (e.g. spinal cord, pancreas, gut, etc.). These cultures are enriched for the first cell type such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin  $\beta$ 3 for neurons, Islet-1 for pancreatic islet cells).
2. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, remove the culture medium and establish a co-culture of the first and second cell types as follows. Rinse the first cell type cultures 3 times with fresh culture medium lacking neuregulin so as to rinse away residual neuregulin. Add back a suspension of the second cell type, from the same tissue of interest as in step 1 in fresh medium lacking neuregulin. The suspension is enriched for the second cell type, such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers.
3. In parallel to step 2, plate the same suspension of cells of the second cell type on the first cell type cultures that have not been treated with neuregulin (control co-cultures).
4. Maintain the first cell type/second cell type co-cultures for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of cellular phenotype of the second cell type such as, but not limited to, cell survival, morphology, production of enzymes and secreted products, etc.
5. Assess the effects of neuregulin. Neuregulin is trophic for the first cell type in a manner that promotes the production of products trophic for the second cell type if:
  - a. The first cell type cultures pre-treated with neuregulin maintain or increase desired aspects of cellular phenotype of the second cell type such as, but not limited to cell survival, morphology, production of enzymes and secreted products, etc.;

and

b. The first cell type cultures that have not been pre-treated with Product A lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

5           The induction by neuregulin of a cell surface-bound or extracellular matrix-bound product, Product A, such that Product A affects a third cell type, can also be tested as in Method II. In steps 2-4, use a suspension of the third cell type rather than the second cell type such that preferably greater than 90% of the cells can be demonstrated to be the third cell type through the use of immunocytochemical and/or  
10 enzymatic markers.

          Described below are methods for detecting the activities of a neuregulin that induces neuronally-associated tissues to produce a neurotrophic product or product(s) (Product A):  
15

**Method III -**

The use of separate cultures of neurons and neuronally associated tissues, to demonstrate that neuregulin induces a neuronally associated tissue (the first cell type) to produce a secreted product that is trophic for neurons (the second cell type).

1. Establish neuron-free cultures of neuronally-associated cell types (e.g. glia, fibroblasts). These cultures are enriched for a single cell type (the first cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. S-100 for peripheral glia (K. R. Jessen and R. Mirsky, Schwann cell: early lineage, regulation of proliferation and control of myelin formation. *Curr. Op. Neurobiol.* (1992) 2:575-581), fibronectin for fibroblasts (K. M. Yamada, Cell surface interactions with extracellular materials. *Ann. Rev. Biochem.* (1983) 52:761-799)).
2. Establish cultures of neurons from the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column). These cultures are enriched for neurons (the second cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin  $\beta 3$  for all neurons, choline acetyltransferase for cholinergic neurons (J.C. Martinou, A. L. V. Thai, G. Cassar, F. Roubinet, and M. J. Weber, Characterization of two factors enhancing choline acetyltransferase in cultures of purified rat motoneurons. *J. Neurosci.* (1989) 9:3645-3656)).
3. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, collect the conditioned culture medium, remove debris by centrifugation (200 g, 10 minutes) and filtration (nylon filter, 0.22 mm pore size).
4. Replace or supplement the medium of the second cell type cultures with media prepared as in step 3. Include among these medium samples, medium that has been conditioned by the first cell type cultures in the absence of neuregulin (control conditioned medium). Include among the medium samples, media containing neuregulin that have not been conditioned by the first cell type cultures (non-conditioned medium).
5. Maintain the second cell type cultures as described in step 4 for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of

neuronal phenotype such as, but not limited to cell survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.

6. Assess the effects of neuregulin. Neuregulin is trophic for neuronally-associated  
5 tissues in a manner that promotes the production of neurotrophic products if:
- a. Medium conditioned by the first cell type cultures in the presence of neuregulin maintains or increases desired aspects of neuronal phenotype such as, but not limited to cell survival, increased neurite (axon or dendrite) outgrowth, neurotransmitter synthesis, etc.;
- 10 b. equal volumes of control conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.);
- and
- c. equal volumes of non-conditioned medium lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

15

If Product A is not secreted, but is bound to the surface of the first cell type, or is bound to insoluble extracellular matrix associated with the first cell type, an alternative procedure is to be used:

**Method IV -**

5 The use of separate cultures of neurons and neuronally associated tissues, to demonstrate that neuregulin induces a neuronally associated tissue (the first cell type) to produce a substance on its surface that is trophic for neurons.

1. Establish neuron-free cultures of neuronally-associated cell types (e.g. glia, fibroblasts). These cultures are enriched for a single cell type (the first cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. S-100 for peripheral glia, fibronectin for fibroblasts).  
10
2. Expose the first cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, remove the culture medium and establish a co-culture of the first cell type and neurons (the second cell type) as follows. Rinse the first cell type cultures 3 times with fresh culture medium lacking neuregulin so as to rinse away residual neuregulin. Add back a suspension of neurons from the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column) in fresh medium lacking neuregulin.  
15  
20 The suspension is enriched for the second cell type such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin  $\beta$ 3 for all neurons, choline acetyltransferase for cholinergic neurons).
- 25 3. In parallel to step 2, plate the same suspension of the second cell type cells on the first cell type cultures that have not been treated with Product A (control co-cultures).
4. Maintain the first cell type/second cell type co-cultures for varying periods of time preferably greater than 1 day and less than 7 days. Assess various aspects of neuronal phenotype such as, but not limited to cell survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.  
30
5. Assess the effects of neuregulin. Neuregulin is trophic for the first cell type in a manner that promotes the production of products trophic for the second cell type if:  
35
  - a. The first cell type cultures pre-treated with neuregulin maintain or increase desired aspects of neuronal phenotype such as, but not limited to cell survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.;



and

b. The first cell type cultures that have not been pre-treated with Product A lack the activity described in criterion (a.), or demonstrate lesser degrees of the activity described in criterion (a.).

5

If cultures of non-neuronal cells of interest greater than 90% pure have not been established, the following method can be used:

**Method V -**

The use of a mixed culture, to demonstrate that neuregulins induce the first cell type (neuronally associated cell types) to produce a product (Product A) that affects the  
5 second cell type.

1. Establish undissociated, explant cultures of the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column). These cultures are not enriched for various cell types and are constituted of both neurons (the second cell type) and  
10 neuronally-associated cell types (the first cell type) as demonstrated through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin  $\beta 3$  for all neurons, acetylcholinesterase for cholinergic neurons, S-100 for peripheral glia, fibronectin for fibroblasts).
- 15 2. Expose explant cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, assess various aspects of neuronal phenotype such as, but not limited to neuron survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.
- 20 3. Establish cultures of neurons from the neuronal tissue of interest (e.g. superior cervical ganglion, spinal motor column). These cultures are enriched for neurons (the second cell type) such that preferably greater than 90% of the cells can be demonstrated to be the same cell type through the use of immunocytochemical and/or enzymatic markers (e.g. tubulin  $\beta 3$  for all neurons, choline acetyltransferase for cholinergic  
25 neurons).
4. Expose the second cell type cultures to varying doses of neuregulin for varying periods of time, preferably greater than 1 hour and less than 7 days. At the end of the culture period, assess various aspects of neuronal phenotype such as, but not limited to  
30 neuron survival, neurite (axon or dendrite) outgrowth, neurotransmitter phenotype, etc.
5. Assess the effects of neuregulin. Neuregulin is trophic for neuronally-associated tissues in a manner that promotes the production of neurotrophic products if:
  - a. in explant cultures the presence of neuregulin maintains or increases desired  
35 aspects of neuronal phenotype such as, but not limited to neuron survival, neurite (axon or dendrite) outgrowth, neurotransmitter synthesis, etc.;

and

- b. in the second cell type cultures, neuregulin lacks the activity described in criterion (a.), or demonstrates lesser degrees of the activity described in criterion (a.)

## EXAMPLES

### Example 1

#### 5    **The Effect of Recombinant Human Glial Growth Factor 2 on Sympathetic Ganglion Outgrowth in an *In Vitro* Model of Peripheral Nerve Gap Entubulation**

##### **Purpose**

10            One approach to the repair of injuries in which a peripheral nerve has been severed is to suture the nerve endings together via a biocompatible tube, a procedure referred to as entubulation. The tube may be filled with various agents thought to improve the growth and regeneration of the nerve. Peripheral nerves contain a variety of cell types: neurons (or more appropriately, the axons emanating from neuron cell  
15    bodies located in the spinal cord and associated ganglia), Schwann cells (peripheral glia), fibroblasts, and resident macrophages. Axons regenerate from the side of the nerve gap proximal to the spinal cord and associated ganglia; other cell types contribute to regeneration by migrating in from both sides of the gap and proliferating.

20            In an effort to devise an *in vitro* model of entubulation, a technique was developed in which fragments of the rat superior cervical ganglion (SCG) are cultured in segments of surgical tubing used in whole animal models of peripheral nerve entubulation. SCG neurons are homogenous in their trophic requirements and project axons exclusively through peripheral nerves; SCG fragments also contain Schwann  
25    cells, fibroblasts, and macrophages. In this model the SCG fragments serve as surrogate proximal nerve endings, and the outgrowth of axons and supporting cell types can be observed in a simplified environment. The focus of this example was to examine the effects of rhGGF2 on Schwann cell and axon behavior in this *in vitro* model of peripheral nerve entubulation.

30

##### **Methods and Materials**

###### ***Tube Preparation***

35            Tubing used for this study was polyethylene tubing with an internal diameter of 1.19 mm and outer diameter of 1.70 mm (Intramedic®: Becton Dickinson and Company; Parsippany, New Jersey). A length of tubing somewhat longer than actually needed was cut in a sterile tissue culture hood, immersed in 70% ethanol, and flushed repeatedly with 70% ethanol using a syringe with a 19-gauge needle. After soaking the

tubing for approximately 30 minutes, it was flushed again with air, and allowed to dry in the hood. After drying, the tubing was cut into 10 mm segments with a sterile scalpel, and stored in a sterile Petri dish.

## 5 *Culture Medium*

Culture medium was made freshly on the day of culture assembly. All components were kept cold (either 4° C or on ice), as was the final solution until culture assembly was completed.

10	Sterile Water	2.60
	Sodium bicarbonate (2% w/v)	1.50
	Penicillin/Streptomycin stock*	0.15
	L-Glutamine (200 mM)	0.15
	Fetal Bovine Serum**	0.75
15	Sodium hydroxide (0.1 M)	0.90
	10x Medium***	1.50
	Collagen solution****	<u>7.40</u>
	TOTAL	15.00 ml

20

\* 5000 units/ml penicillin, 5 mg/ml streptomycin

\*\* heat inactivated (Hyclone; Logan, UT)

\*\*\* one packet of low glucose Dulbecco's Modified Essential Medium (DMEM: Gibco/BRL; Grand Island, NY) meant to make 1 liter of medium dissolved in 100 ml of

25 sterile water

\*\*\*\* 3 mg/ml Vitrogen-100® (Celtrix Pharmaceuticals; Santa Clara, CA)

Medium was used as is, or was supplemented with rhGGF2 as indicated.

### *Tube Culture Assembly*

A schematic diagram of culture assembly is shown in Figure 1. SCGs were dissected from postnatal day 0-2 rats on the day of assembly, cleaned of connective tissues and proximal nerve stumps, bisected, and stored in physiological saline at 4° till  
5 needed. Since the collagen-containing medium gels at room temperature or higher, it is necessary to assemble the cultures in 4° cold room. Working with watchmaker's forceps under a dissection microscope at total magnification of 8x, individual segments of cleaned tubing were picked up and filled with culture medium using a syringe with a  
10 27-gauge needle. A single piece of bisected SCG was then placed at the very end of each tube, and each tube placed in an individual well of a 24-well tissue culture plate. Only the central eight wells of a 24-well plate were used for tube placement, and the remaining wells were filled with sterile water to maintain plate humidity. The plate was then placed in a 37°, 10% carbon dioxide incubator. After allowing the cultures to gel and equilibrate with the incubator atmosphere, the plates were sealed with paraffin film  
15 to further protect the culture assemblies from dehydration, and returned to the incubator until preparation for immunocytochemistry and analysis.

### *Immunocytochemistry*

After 2, 5, and 10 days *in vitro*, the contents of individual tube cultures were  
20 extruded into phosphate buffered saline (PBS) using a PBS-filled syringe with a blunt-ended 18-gauge needle. The collagen gels retained structural integrity and were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. After 3 washes with PBS, the cultures were blocked in 1% goat serum/0.1% Triton X-100 in PBS for 30 minutes. After blocking, the solution was changed to 1% goat serum in PBS (GPBS)  
25 containing 1:4 rabbit anti-S-100 (a Schwann cell marker; Incstar; Stillwater, MN) and 1:400 mouse anti-tubulin  $\beta 3$  (an axon marker; Sigma; St. Louis, MO). After incubating the samples in primary antibody for 1 hour at room temperature, they were washed 3 times with PBS, and incubated for an additional hour in GPBS containing 1:200 peroxidase-conjugated goat anti-mouse immunoglobulin, and 1:200 alkaline  
30 phosphatase-conjugated goat anti-rabbit immunoglobulin (Pierce Chemical; Rockford, IL). The samples were then washed 3 times with PBS, and the stains developed. The S-100 was first developed using stable pre-mixed NBT/BCIP (Gibco/BRL) to yield a blue stain, and after rinsing with PBS, the tubulin  $\beta 3$  was developed using 3-amino-9-ethylcarbazole (AEC; Sigma) per manufacturer's directions. After final rinsing with  
35 PBS, the samples were mounted on microscope slides using aqueous mounting medium. After the mounting medium dried, the cellular outgrowth could be analyzed.

### *Scoring and Analysis*

As schematized in Figure 2, a grid reticule was placed in the microscope ocular, and at a total magnification of 160x, the total number of S-100<sup>+</sup> Schwann cells in each column (referred to as "bins") was counted. Each bin has a width of 50 mm as  
5 determined using a stage micrometer. And as noted, the number of tubulin  $\beta$ <sup>3+</sup> neurites intersecting every vertical line was also counted. The actual grid was not large enough to cover the entire length of cellular outgrowth and was shifted along as needed by translational movement of the microscope stage. All data points represent the average  $\pm$  the standard error of the mean (n = 6 to 7 for every data point).

### **Results**

First shown is the analysis of Schwann cell number as a function of distance from the SCG explant (Figure 3A-D). It is clear that the presence of rhGGF2 affects the  
15 behavior of Schwann cells relative to the control condition. There does not appear to be any difference among the 3 doses of rhGGF2. Generally, by 5 days in rhGGF2, there is a large increase in the number of Schwann cells proximal to the explant, but the Schwann cells appear to have moved only about as far as they have in the control case (somewhat further at the highest dose). By 10 days in rhGGF2 the overall number of  
20 Schwann cells has decreased, but the cells still present have definitely migrated farther than in the absence of rhGGF2. In the absence of rhGGF2, the controls look no different between days 5 and 10. The total number of Schwann cells in the various conditions is shown in Figure 4. Again, there is a decrease in cell number at day 10, but there is no obvious difference between the different doses of rhGGF2. The day 10 tubes  
25 contain more debris, and this is probably due to cell death. This is due to the culture situation since 10 days appears to be the longest that one can maintain these tube cultures without overt signs of dehydration and nutrient depletion in the limited volume of culture medium (approximately 10  $\mu$ l per tube).

A difference is apparent when neurites are scored in the various doses of  
30 rhGGF2 (Figure 5). At doses of rhGGF2 greater than or equal to 50 ng/ml, a profound increase takes place in the number of neurites and the extent to which they have grown away from the explant.

### **Discussion and Conclusions**

35 This study demonstrates that the dose range in which there are observable effects on Schwann cell proliferation and emigration from the explant is different from that which causes a major increase in neurite outgrowth. In the case of the former, it

appears that the effect has plateaued at the lowest dose tested, 5 ng/ml. As for the requirement of  $\geq 50$  ng/ml rhGGF2 to boost neurite regeneration, there are two possible mechanisms to account for this. One is that rhGGF2 is acting directly upon the neurons, and the other is that rhGGF2 induces a non-neuronal cell type to produce a neurite promoting factor (e.g. NGF, secreted extracellular matrix proteins, proteases, and/or protease inhibitors). As is demonstrated in Example 2, the first hypothesis is not likely since rhGGF2 has no effect upon neuronal survival or outgrowth in low density cultures of dissociated SCG neurons. This lack of a direct effect on neurons implies that the rhGGF2 promotion of neurite outgrowth is due to rhGGF2 induced production of neurite promoting factors by non-neuronal cells.



## Example 2

### The Promotion of Axon Outgrowth by Recombinant Human Glial Growth Factor 2 is Not Due to a Direct Effect on Neurons

5

#### Purpose

As demonstrated in Example 1, rhGGF2 not only promotes Schwann cell proliferation and migration in an *in vitro* model of peripheral nerve entubulation, but also promotes robust axonal outgrowth. To test whether this may be due to direct effects of rhGGF2 on SCG neurons, low density cultures of dissociated SCG neurons were established in which the effects of rhGGF2 could be examined. SCG neurons are normally dependent upon nerve growth factor (NGF) for survival, so rhGGF2 was tested for direct neuronal effects in the simultaneous presence of a wide range of NGF concentrations.

15

#### Methods and Materials

##### *Cell Culture*

20

SCGs were dissected from postnatal day 0-2 rats, cleaned of connective tissue and proximal nerve stumps, and dissociated by enzymatic digestion and trituration. Enzymatic digestion was performed using 1 mg/ml trypsin (Sigma; St. Louis, MO) and 1 mg/ml collagenase (Boehringer-Mannheim; Indianapolis, IN) in calcium- and magnesium-free Hanks's Balanced Salt Solution (HBSS; Gibco/BRL; Grand Island, NY), for 1 hour at 37° C. Trituration was performed using a flame-polished Pasteur pipet. Dissociated neurons were taken up in plating medium and pre-plated in tissue culture dishes for 1 hour to remove the majority of the rapidly adherent, non-neuronal cells. Plating medium consisted of low glucose DMEM (Gibco/BRL) supplemented with glutamine, penicillin/streptomycin, and fetal bovine serum to the same concentrations as described in Example 1. Non-adherent cells (primarily neurons), were pelleted by centrifugation and resuspended in plating medium. These cells were finally plated at a density of 5000 cells per well in collagen-coated, 24-well plates such that the cells were exposed to a 2-dimensional dose-response matrix of NGF and rhGGF2 (Figure 6A). Plates were set up in duplicate on 2 different dates; at the completion of both experiments N = 4 for each of the 24 conditions. The cultures were only allowed to progress for 2 days since this is a time frame in which any

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contaminating Schwann cells could have only undergone a single doubling, and sufficient for ascertaining whether the factors have promoted neuronal survival.

#### *Staining and Scoring of the Cultures*

5

After 2 days, the cultures were fixed and stained for tubulin  $\beta 3$  as described in Example 1. The tubulin  $\beta 3$ -positive, neurite-bearing cells were counted in each well at a total magnification of 100x. Due to meniscus effects, and incubator vibration during the initial plating period, cells tend to preferentially concentrate in the center of the well. Thus in order to get a reasonably representative count of cell number, 5 fields per well were counted: the center most field and four flanking fields (Figure 6B). This manner of counting was used on all wells and is sufficiently consistent for the purpose of comparing the effects of different growth factor concentrations and combinations. The number of cells counted in every well was normalized such that the average number counted in the wells that received 0 ng/ml rhGGF2, and 100 ng/ml NGF equals a value of 100.

#### **Results**

20

It is clear from the results presented in Figure 7, that rhGGF2 has no direct effect on the survival of SCG neurons. All surviving neurons exhibited robust axon outgrowth, and there was no noticeable effect on the extent of axon outgrowth. As expected in the absence of rhGGF2, the number of neurons reaches a plateau at 10 ng/ml. The presence or absence of rhGGF2 appears to make no difference at the 3 doses tested.

25

#### **Discussion and Conclusions**

In light of the results presented in Example 1, it was necessary to examine whether the effect of rhGGF2 on axon outgrowth could be attributed to a direct effect of rhGGF2 on the neurons in question. The results of Example 2 make it clear that this is not the case. Thus one must conclude that the effect of rhGGF2 on axon outgrowth observed in the tube paradigm is due to a "bystander effect" rather than a direct action on the neurons. Thus rhGGF2 can promote the healing response of injured neurons by inducing the production of neurite promoting factors by non-neuronal support cells.

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**Example 3****Increase in myelinated axon growth in an animal model of peripheral nerve injury mediated by a neuregulin**

5

An animal model of peripheral nerve repair was used to test the ability of a neuregulin (rhGGF2) to increase the number of regenerating axons. The rationale is that added rhGGF2 will induce increases in Schwann cell (the first cell type) numbers as well as increases in the levels of trophic factors (Product A) produced by Schwann cells that, in turn, will affect a second cell type, the regenerating axons (the second cell type) as measured by increases in the number of myelinated axons (response ).

15 Fisher 344 rats (male, 195-250g) were surgically prepared and one sciatic nerve was transected resulting in a 10mm gap. Polyethylene guide tubes (13mm in length, 1.1mm internal diameter) were prepared. These tubes contained a flat sliver of a collagen coated Immobilon filter (1.0x10mm) containing immobilized rhGGF2 and were prepared as described in U.S. Patent Application Serial No. 08/293,465, filed on August 19, 1994, hereby incorporated by reference (Immobilon: Millipore, Corp., Bedford, MA). The strips were inserted into the lumen of the guide tubes. rhGGF2 was used at a concentration of 162  $\mu\text{g}/\mu\text{L}$  (in phosphate buffered saline), 2.5  $\mu\text{L}$  of this solution was added per strip. Control tubes were prepared containing collagen coated Immobilon strips treated with phosphate buffered saline alone. Tubes were secured with a single suture at the proximal and distal ends after filling the lumen with physiological saline and sealing the ends with vaseline.

25

Animals (10 rhGGF2 treated, 10 controls) were sacrificed at 28 days and the section of sciatic nerve containing the tube was excised, the nerve was removed from the tube and a cross section was taken from the mid point of the tube and prepared for histological analysis. The material was fixed in 4% paraformaldehyde and 2% glutaraldehyde for 24h and then post fixed in 2% osmium tetroxide and embedded in glycomethacrylate. One micron cross sections were taken and stained with 1 $\mu\text{M}$  toluidine blue.

35 A histological analysis of a section from the mid point of the tube was performed and measurements were made of the total number of myelinated axons in a section and the total endoneurial area in each section. The data are shown in Figures 8A and 8B.

The rhGGF2 treated animals showed a 2.1 fold increase in the number of myelinated axons over the control animals.

5 The results of this study demonstrate a positive effect of exogenously added rhGGF2 on the growth of myelinated axons. In consideration of the data discussed in Example 1 where rhGGF2 acts on Schwann cells to induce the synthesis of products that are trophic for regenerating axons in an *in vitro* paradigm it is concluded that a similar mechanism is responsible for the rhGGF2 mediated enhancement of the growth of axons *in vivo*.

**Claims:**

1. A method of affecting cellular communication in a vertebrate, comprising  
5 administration of a neuregulin to said vertebrate wherein said neuregulin interacts with a first cell type, resulting in production of a Product A of said first cell type and said Product A affects a function of a second cell type.
2. A method of Claim 1, wherein, said affect in function of said second cell type results  
10 in the production of a Product B of said second cell type and said Product B affects the function of said first cell type or a third cell type.
3. A method of affecting cellular communication in a vertebrate, comprising  
15 administration of a neuregulin-producing cell to said vertebrate wherein a neuregulin is produced and said neuregulin interacts with a first cell type, resulting in production of a Product A of said first cell type and said Product A affects a function of a second cell type.
4. A method of Claim 3, wherein, said affect in function of said second cell type results  
20 in the production of a Product B of said second cell type and said Product B affects the function of said first cell type or a third cell type.
5. A method of affecting cellular communication in a vertebrate, comprising  
25 administration of DNA encoding a neuregulin to said vertebrate wherein DNA is incorporated into a genome of a cell and said DNA is expressed in said cell resulting in the production of said neuregulin which interacts with a first cell type, resulting in production of a Product A of said first cell type and said Product A affects a function of a second cell type.
- 30 6. A method of Claim 5, wherein, said affect in function of said second cell type results in the production of a Product B of said second cell type and said Product B affects the function of said first cell type or a third cell type.
7. A method of Claim 1 wherein said vertebrate is a human.
- 35 8. A method of Claim 1 wherein said first cell type is a nervous system support cell.
9. A method of Claim 8 wherein said nervous system support cell is a Schwann cell.

10. A method of Claim 1 wherein said first cell type is a neuron.
11. A method of Claim 1 wherein said first cell type is a muscle cell.
- 5 12. A method of Claim 1 wherein said Product A is a neurotrophic agent.
13. A method of Claim 1 wherein said Product A is a matrix molecule.
- 10 14. A method of Claim 1 wherein said Product A is a protease.
15. A method of Claim 1 wherein said Product A is a protease inhibitor.
16. A method of Claim 1 wherein said second cell type is a nervous system cell.
- 15 17. A method of Claim 1 wherein said second cell type is a muscle cell.
18. A method of Claim 1 wherein said affect in function of said second cell type is differentiation.
- 20 19. A method of Claim 1 wherein said affect in function of said second cell type is mitosis.
- 25 20. A method of Claim 1 wherein said affect in function of said second cell type is survival.
21. A method of Claim 2 wherein said Product B is a neurotrophic agent.
22. A method of Claim 2 wherein said Product B is a matrix molecule.
- 30 23. A method of Claim 2 wherein said Product B is a protease.
24. A method of Claim 2 wherein said Product B is a protease inhibitor
- 35 25. A method of Claim 2 wherein said Product B is a neuregulin.
26. A method of Claim 25 wherein said neuregulin is rhGGF2.

27. A method of Claim 2 wherein said affect in function of said first cell type or said third cell type is differentiation.
28. A method of Claim 2 wherein said affect in function of said first cell type or said  
5 third cell type is mitosis.
29. A method of Claim 2 wherein said affect in function of said first cell type or said third cell type is survival.
- 10 30. A method of Claim 2 wherein said third cell type is a nervous system cell.
31. A method of Claim 2 wherein said third cell type is a muscle cell.
32. A method of treating a neurological disorder in a mammal, comprising  
15 administration of a therapeutically effective amount of a neuregulin to said mammal wherein said neuregulin interacts with a nervous system cell, resulting in the production of a neurotrophic agent which affects the function of a neuron cell type.
33. A method of treating a neurological disorder in a mammal, comprising  
20 administration of a neuregulin producing cell to said mammal wherein said produced neuregulin interacts with a nervous system cell, resulting in the production of a neurotrophic product which affects the function of a neuron cell type.
34. A method of treating a neurological disorder in a mammal, comprising  
25 administration of DNA encoding a neuregulin to said mammal wherein said neuregulin is produced and interacts with a nervous system cell, resulting in the production of a neurotrophic agent which affects the function of a neuron cell type.
35. A method of treating peripheral neuropathy, amyotrophic lateral sclerosis, spinal  
30 muscular atrophy, nerve injury, Alzheimer's Disease, Parkinson's Disease and spinal cord injury comprising the administration of a therapeutically effective amount of a neuregulin wherein said neuregulin interacts with a first cell type, resulting in the production of a Product A of the first cell type and said Product A affects a function of a second cell type.
36. A method of inducing the endogenous production of a product by a cell in a vertebrate comprising administration of a neuregulin to said vertebrate, wherein said cell produces said product.

37. A method of claim 36 wherein said product is a neurotrophic agent.



Figure 1

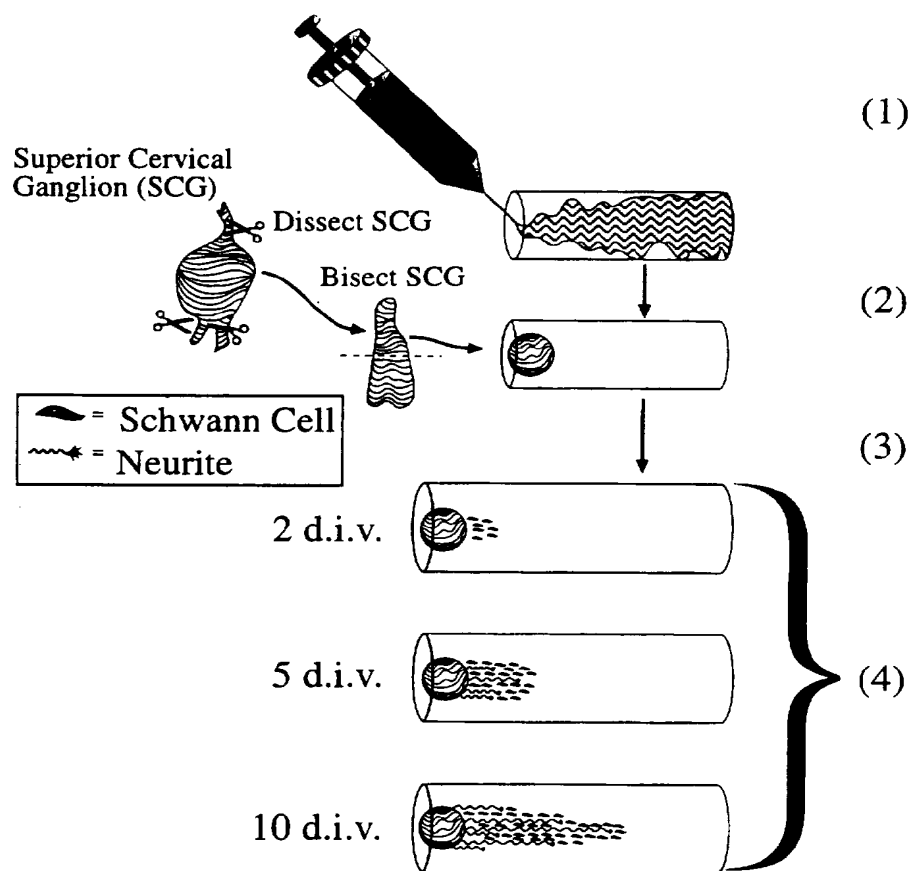


Figure 2

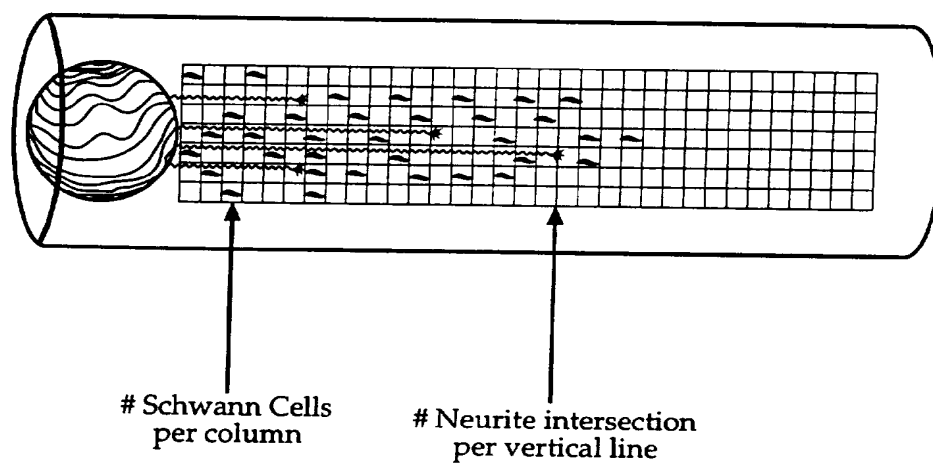


Figure 3A

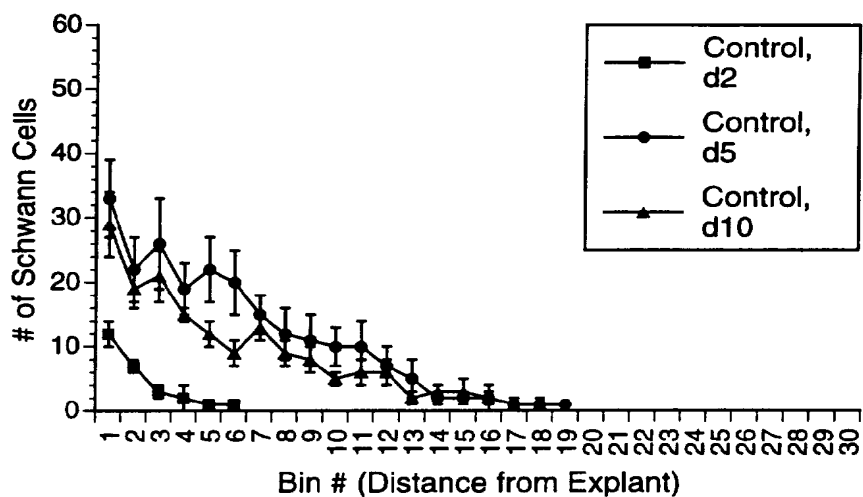


Figure 3B

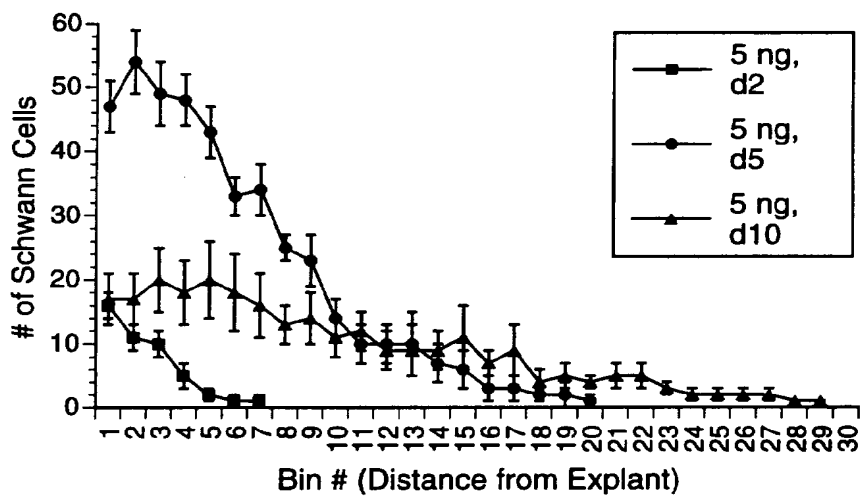


Figure 3C

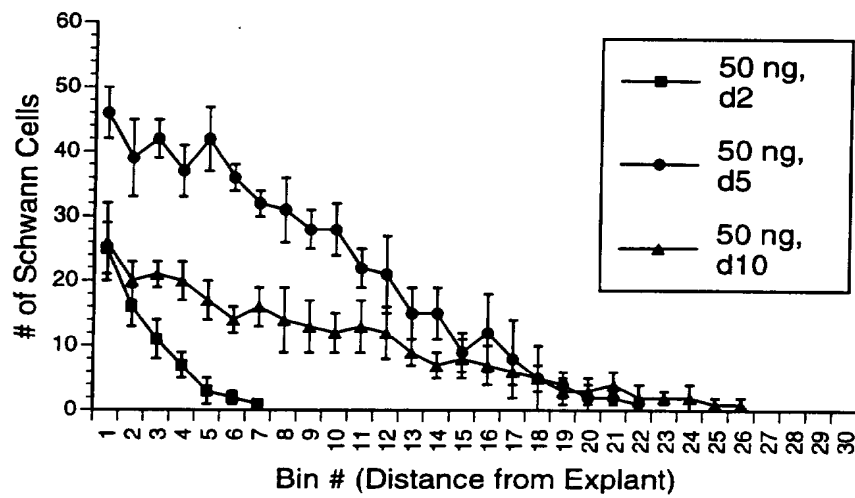


Figure 3D

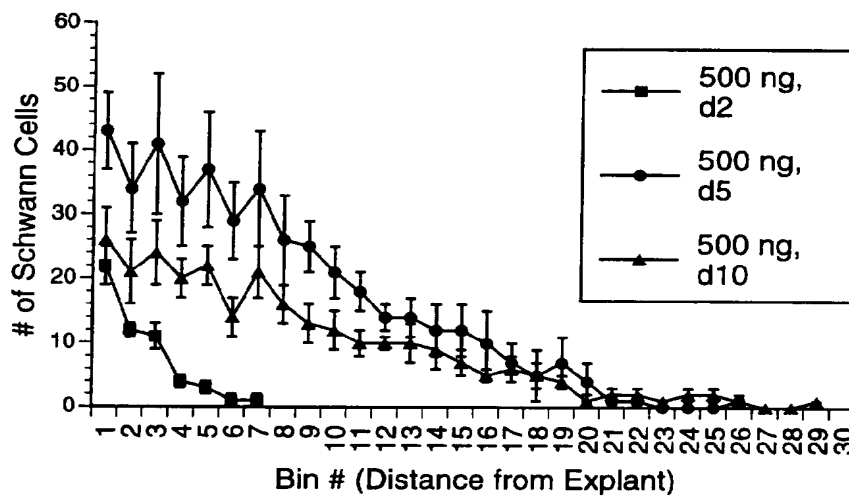


Figure 4

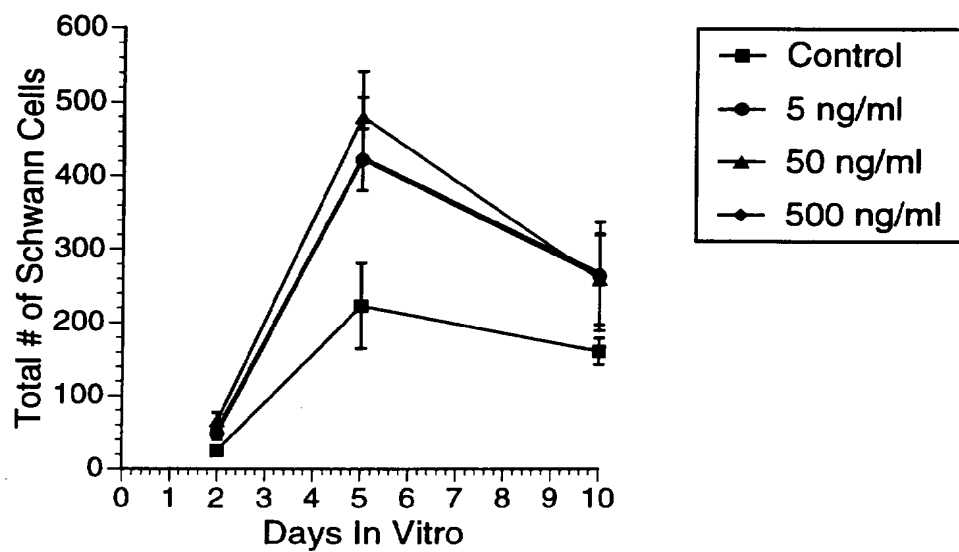


Figure 5

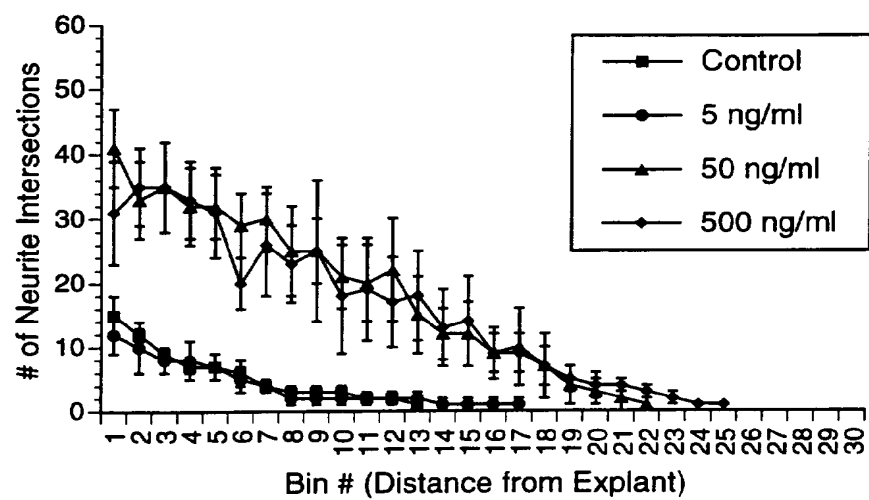


Figure 6A

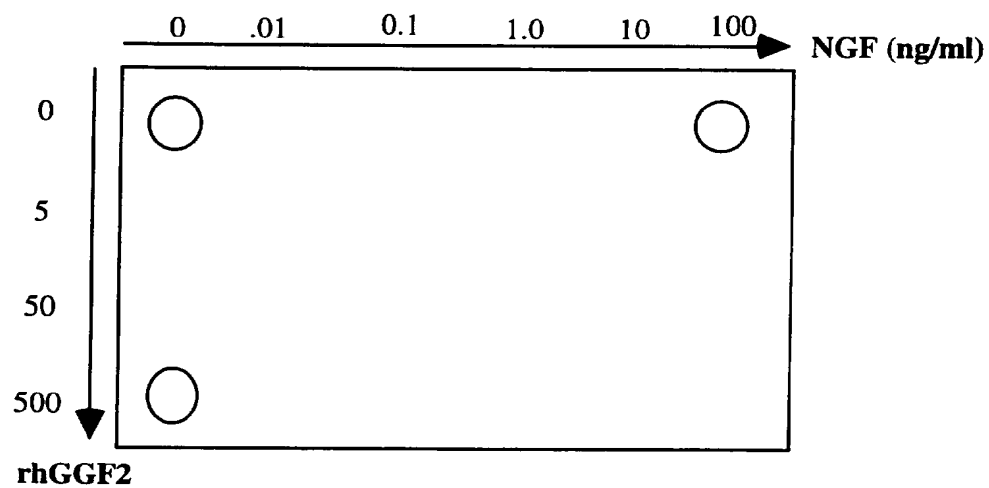


Figure 6B

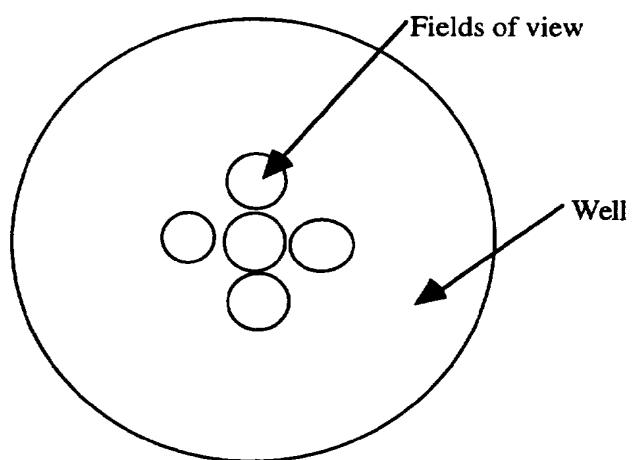


Figure 7

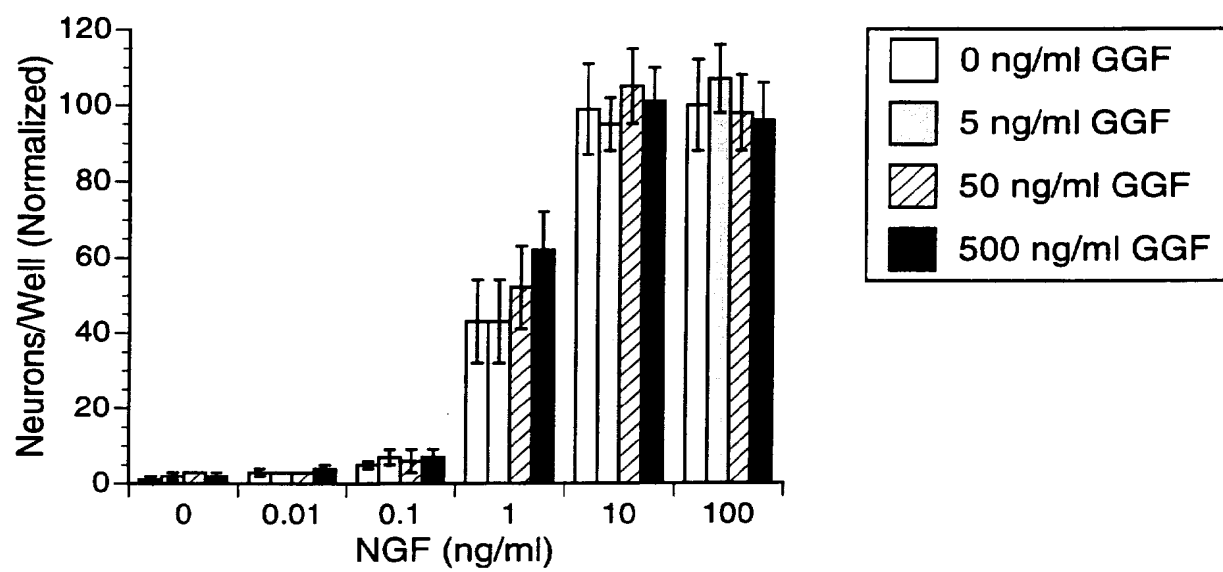




Figure 8A

	Total Myelinated Axons	Total Endoneurial Area	Axon Density
GGF group (n=9)			
GGF2 mean	741	165345( $\mu\text{M}^2$ )	4143(per $\text{mm}^2$ )
GGF2 std. error	200	14809	951
Control group (n=10)			
Control mean	355	130931( $\mu\text{M}^2$ )	2938(per $\text{mm}^2$ )
Control std. error	76	14917	722

Figure 8B

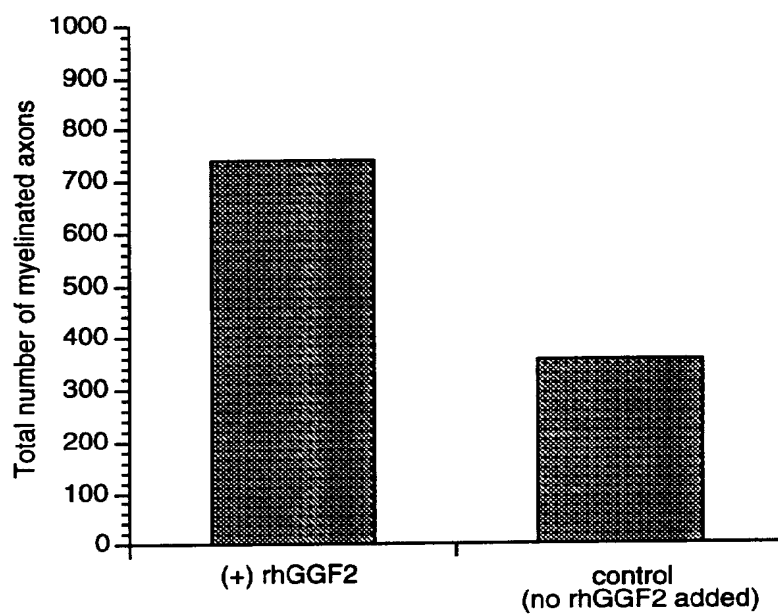


Figure 9

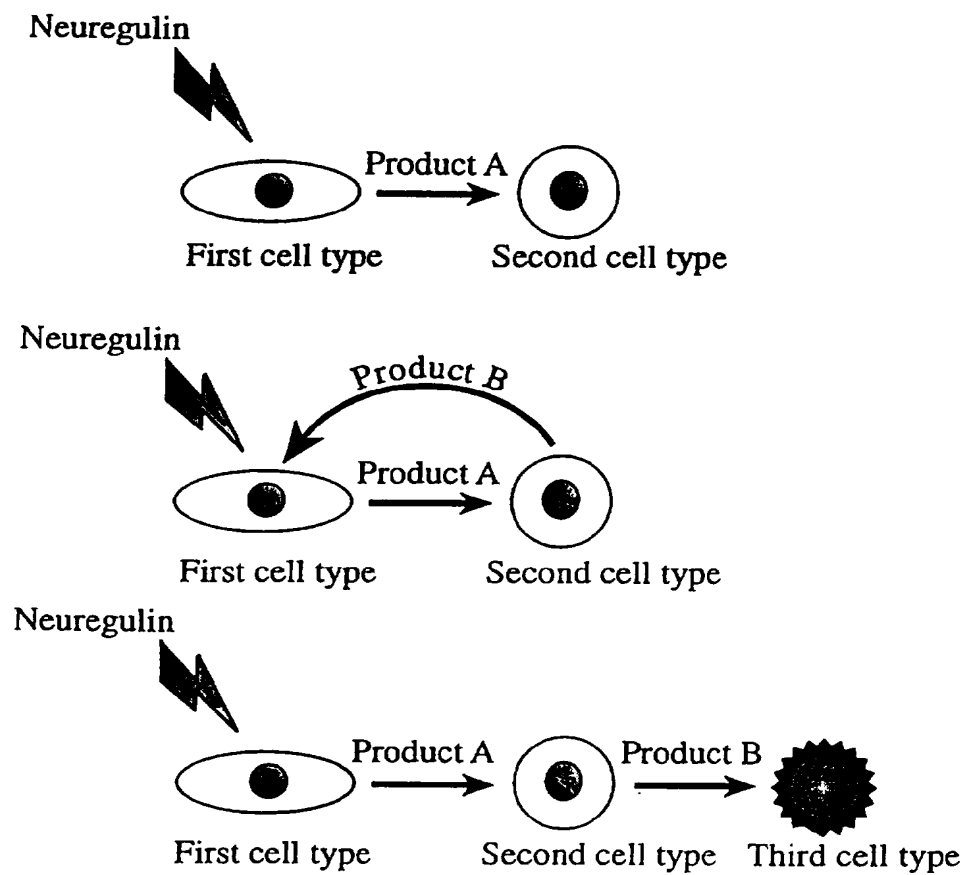


Figure 10

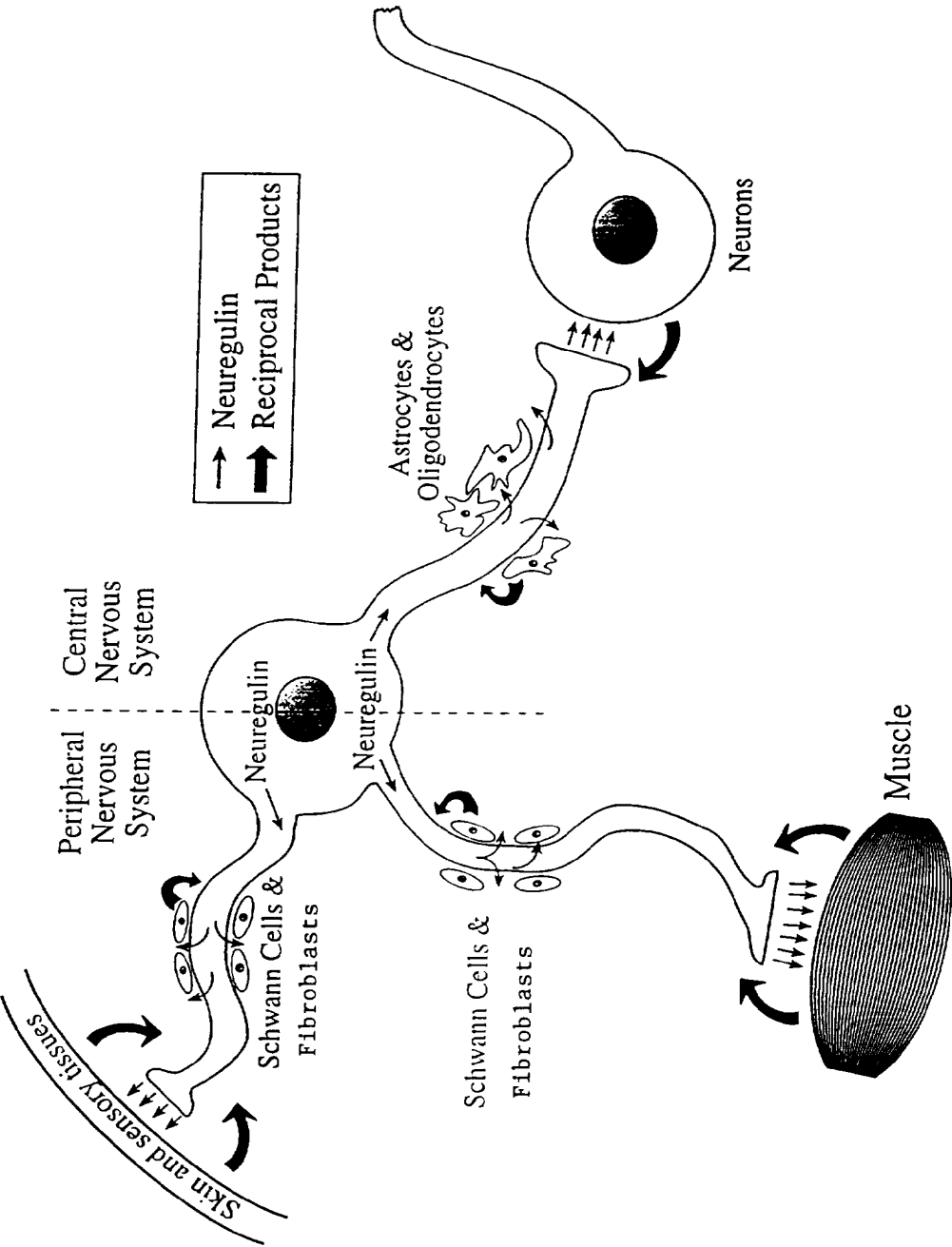


Figure 11 A

CCTGCAG	CAT	CAA	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	55
	His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
	1				5				10						15		
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC		103
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys		
		20					25					30					
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG		151
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu		
	35					40						45					
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC		199
Ala	<u>Asn</u>	<u>Ser</u>	<u>Ser</u>	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro		
	50					55				60							
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG		247
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val		
65				70					75					80			
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG		295
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu		
			85				90							95			
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA		343
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu		
		100				105						110					
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC		391
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	<u>Asn</u>	<u>Gly</u>	<u>Ser</u>	Glu	Leu	Ser		
		115				120					125						
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG		439
Arg	Lys	Asn	Lys	Pro	Glu	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Gly	Lys		
	130					135				140							
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT		487
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr		
145				150					155					160			
ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC		535
Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	<u>Asn</u>	<u>Asp</u>	<u>Ser</u>	Ala	Ser	Ala	<u>Asn</u>		
			165					170				175					
ATC	ACC	ATT	GTG	GAG	TCA	AAC	GGT	AAG	AGA	TGC	CTA	CTG	CGT	GCT	ATT		583
<u>Ile</u>	<u>Thr</u>	Ile	Val	Glu	Ser	Asn	Gly	Lys	Arg	Cys	Leu	Leu	Arg	Ala	Ile		
		180					185					190					
TCT	CAG	TCT	CTA	AGA	GGA	GTG	ATC	AAG	GTA	TGT	GGT	CAC	ACT				625
Ser	Gln	Ser	Leu	Arg	Gly	Val	Ile	Lys	Val	Cys	Gly	His	Thr				
	195					200					205						
TGAATCACGC	AGGTGTGTGA	AATCTCATTG	TGAACAAATA	AAAATCATGA	AAGGAAAAAA												685
AAAAAAAAAA	AATCGATGTC	GACTCGAGAT	GTGGCTGCAG	GTCGACTCTA	GAGGATCCC												744

Figure 11 B

CCTGCAG	CAT	CAA	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	55
	His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
	1				5				10						15		
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC		103
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys		
		20					25					30					
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG		151
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu		
		35					40					45					
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC		199
Ala	Lys	Ser	Ser	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro		
	50					55				60							
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG		247
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val		
65				70					75					80			
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG		295
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu		
			85					90					95				
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA		343
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu		
		100					105					110					
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC		391
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Ser	Glu	Leu	Ser		
		115					120				125						
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG		439
Arg	Lys	Asn	Lys	Gly	Gly	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Gly	Lys		
	130					135					140						
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT		487
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr		
145				150					155					160			
ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC		535
Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn		
			165					170						175			

Figure 11 B'

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA	583
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180 185 190	
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	631
Ser His Leu Val Lys Ser Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
195 200 205	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
210 215 220	
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT	727
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn	
225 230 235 240	
GTG CCC ATG AAA GTC CAA ACC CAA GAA AGT GCC CAA ATG AGT TTA CTG	775
Val Pro Met Lys Val Gln Thr Gln Glu Ser Ala Gln Met Ser Leu Leu	
245 250 255	
GTG ATC GCT GCC AAA ACT ACG TAATGGCCAG CTTCTACAGT ACGTCCACTC	826
Val Ile Ala Ala Lys Thr Thr	
260	
CCTTTCTGTC TCTGCCTGAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC	886
TCCCCTCAGA TTCCTCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT GACTGCCTCT	946
GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC CGTGACTAGT	1006
GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT	1066
ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG GCCTTGAAAA	1126
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TCTAGAG	1193

Figure 11 C

CCTGCAG	CAT	CAA	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	55
	His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
	1				5				10					15			
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC		103
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys		
		20					25					30					
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG		151
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu		
	35					40						45					
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC		199
Ala	<u>Asn</u>	<u>Ser</u>	<u>Ser</u>	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro		
	50					55				60							
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG		247
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gly	Gln	Pro	Gly	Ala	Val		
65				70				75						80			
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG		295
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu		
			85					90					95				
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA		343
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu		
		100					105					110					
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC		391
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	<u>Asn</u>	<u>Gly</u>	<u>Ser</u>	Glu	Leu	Ser		
	115						120				125						
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG		439
Arg	Lys	Asn	Lys	Pro	Glu	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Pro	Lys		
	130					135					140						
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT		487
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr		
145				150					155					160			

Figure 11 C'

ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC	535
Met Cys Lys Val Ile Ser Lys Leu Gly <u>Asn Asp Ser</u> Ala Ser Ala <u>Asn</u>	
165 170 175	
ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA	583
<u>Ile Arg</u> Ile Val Glu Ser <u>Asn Ala Thr</u> Ser Thr Ser Thr Ala Gly Thr	
180 185 190	
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	631
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
195 200 205	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	679
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser <u>Asn Pro Ser</u> Arg Tyr	
210 215 220	
TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC	727
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr	
225 230 235 240	
GTA ATG GCC AGC TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT	775
Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro	
245 250 255	
GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG TTGCCGCATC TCCCCTCAGA TTCCGCCTAG	838
Glu	
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AACACAAGCG ATTGTATGAC TTCTCTGTGC CGTGACTAGT GGGCTCTGAG CTA CTCTCGTAG	958
GTGCGTAAGG CTCCAGTGTT TCTGAAATTG ATCTTGAATT ACTGTGATAC GACATGATAG	1018
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Figure 12

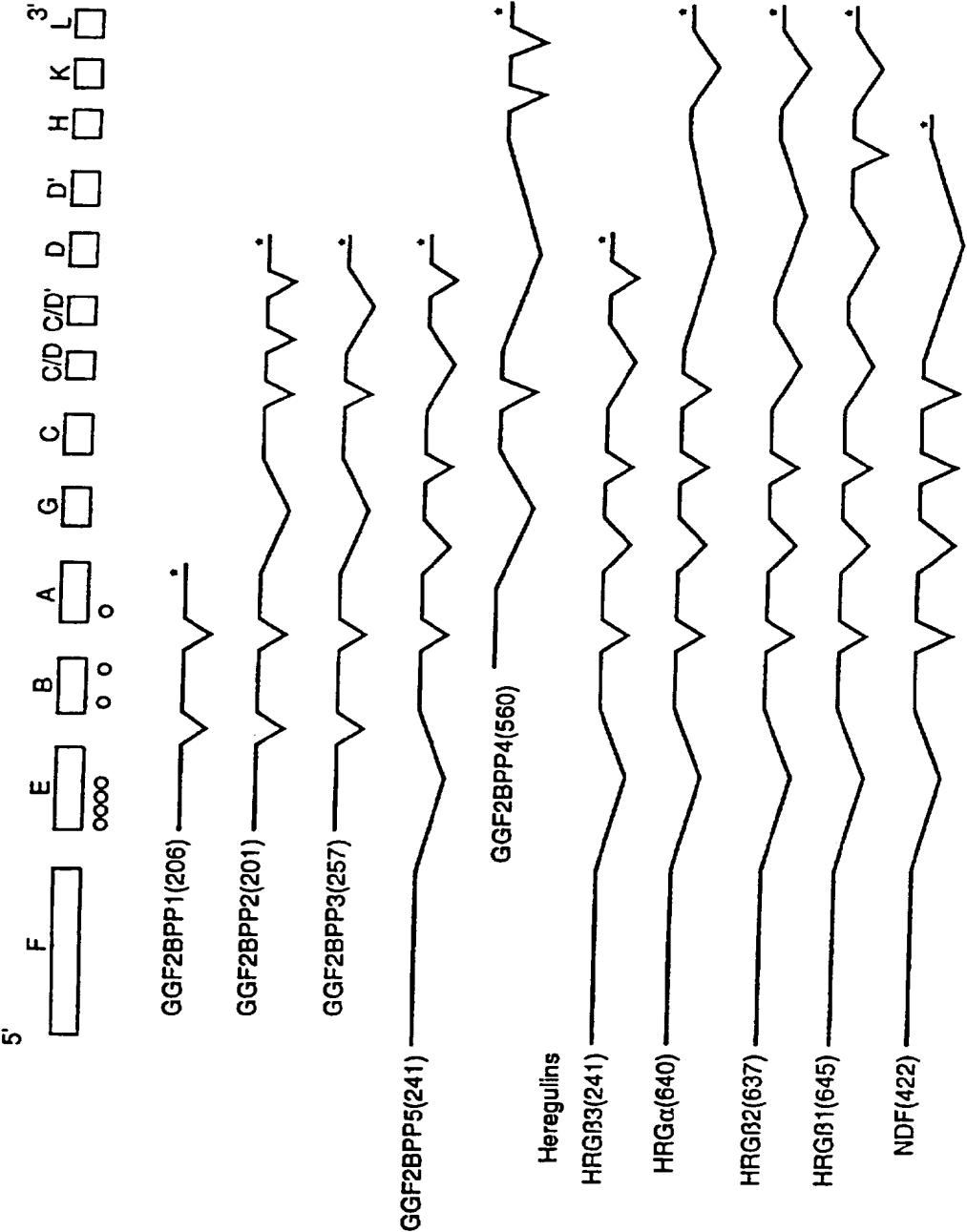


Figure 13 A

## CODING SEGMENT F:

```

AGTTTCCCCC CCCAACTTGT CGGAACCTCTG GGCTCGCGCG CAGGGCAGGA GCGGAGCGGC      60
GGCGGCTGCC CAGGCGATGC GAGCGCGGGC CGGACGGTAA TCGCCTCTCC CTCCTCGGGC      120
TGCGAGCGCG CCGGACCGAG GCAGCGACAG GAGCGGACCG CGGCGGGAAC CGAGGACTCC      180
CCAGCGGCGC GCCAGCAGGA GCCACCCCGC GAGNCGTGCG ACCGGGACGG AGCGCCCGCC      240
AGTCCCAGGT GGCCCGGACC GCACGTTGCG TCCCCGCGCT CCCC GCCGGC GACAGGAGAC      300
GCTCCCCCCC ACGCCGCGCG CGCCTCGGCC CGGTCGCTGG CCCGCCTCCA CTCGCGGGGAC      360
          ||||| | ||||| ||| ||||| ||| ||||| ||| ||||| ||| ||||| |||
          CGCGAG CGCCTCAGCG CGGCCGCTCG CTCTC..CCC CTCGAGGGAC

AAACTTTTCC CGAAGCCGAT CCCAGCCCTC GGACCCAAAC TTGTCGCGCG TCGCCTTCGC      420
||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| ||||| |||
AAACTTTTCC CAAACCCGAT CCGAGCCCTT GGACCAA... ..C TCGCCTGCGC

                                Met Ser Glu Arg Arg
CGGGAGCCGT CCGCGCAGAG CGTGCACTTC TCGGGCGAG ATG TCG GAG CGC AGA      474
||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| ||||| |||
CGAGAGCCGT CCGCGTAGAG CGCTC.CGTC TCCGGCGAG ATG TCC GAG CGC AAA
                                K

Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly
GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG      522
||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| ||||| ||| ||||| |||
GAA GGC AGA GGC AAA GGG AAG GGC AAG AAG AAG GAG CGA GGC TCC GGC
          R                                K                                E

Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala
AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA G      559
||| ||||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
AAG AAG CCG GAG TCC GCG GCG GGC AGC CAG AGC CCA G
          E          S

```

Figure 13 B

## CODING SEGMENT E:

CC CAT CAN GTG TGG GCG GCG AAA GCC GGG GGC TTG AAG AAG GAC TCG	47
His Gln Val Trp Ala Ala Lys Ala Gly Gly Leu Lys Lys Asp Ser	
1 5 10 15	
CTG CTC ACC GTG CGC CTG GGC GCC TGG GGC CAC CCC GCC TTC CCC TCC	95
Leu Leu Thr Val Arg Leu Gly Ala Trp Gly His Pro Ala Phe Pro Ser	
20 25 30	
TGC GGG CGC CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC	143
Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro	
35 40 45	
GAG GCC AAC AGC AGC GGC GGG CCC GGC CGC CTT CCG AGC CTC CTT CCC	191
Glu Ala Asn Ser Ser Gly Gly Pro Gly Arg Leu Pro Ser Leu Leu Pro	
50 55 60	
CCC TCT CGA GAC GGG CCG GAA CCT CAA GAA GGA GGT CAG CCG GGT GCT	239
Pro Ser Arg Asp Gly Pro Glu Pro Gln Glu Gly Gly Gln Pro Gly Ala	
65 70 75	
GTG CAA CGG TGC G	252
Val Gln Arg Cys	
80	

Figure 13 C

## CODING SEGMENT B:

Leu Pro Pro Arg Leu Lys Glu His Lys Ser Gln Glu Ser Val Ala Gly	48
CCT TGC CTC CCC GCT TGA AAG AGA TGA AGA GTC AGG AGT CTG TGG CAG	
CCT TGC CTC CCC GAT TGA AAG AGA TGA AAA GCC AGG AAT CGG CTG CAG	
Q A	
Ser Lys Leu Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu	96
GTT CCA AAC TAG TGC TTC GGT GCG AGA CCA GTT CTG AAT ACT CCT CTC	
GTT CCA AAC TAG TCC TTC GGT GTG AAA CCA GTT CTG AAT ACT CCT CTC	
Lys Phe Lys Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys	144
TCA AGT TCA AGT GGT TCA AGA ATG GGA GTG AAT TAA GCC GAA AGA ACA	
TCA GAT TCA AGT GGT TCA AGA ATG GGA ATG AAT TGA ATC GAA AAA ACA	
R N N	
Pro Gly Asn Ile Lys Ile Gln Lys Arg Pro Gly	178
AAC CAC AAA ACA TCA AGA TAC AGA AAA GGC CGG G	
AAC CAC AAA ATA TCA AGA TAC AAA AAA AGC CAG G	
K	

Figure 13 D

## CODING SEGMENT A:

Lys	Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly			46
G	AAG	TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA		
G	AAG	TCA	GAA	CTT	CGC	ATT	AAC	AAA	GCA	TCA	CTG	GCT	GAT	TCT	GGA		
							N										
Glu	Tyr	Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser		94
GAA	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT		
GAG	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT		
Ala	Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Ala								122
GCC	AAC	ATC	ACC	ATT	GTG	GAG	TCA	AAC	G								
GCC	AAT	ATC	ACC	ATC	GTG	GAA	TCA	AAC	G								

Figure 13 E

## CODING SEGMENT A':

TCTAAAACTA	CAGAGACTGT	ATTTTCATGA	TCATCATAGT	TCTGTGAAAT	ATACTTAAAC			60									
CGCTTTGGTC	CTGATCTTGT	AGG	AAG	TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG			110			
			Lys	Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala						
			1				5										
TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA		158
Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Cys	Lys	Val	Ile	Ser	Lys	Leu		
10					15					20					25		
GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC	ATC	ACC	ATT	GTG	GAG	TCA	AAC	GGT		206
Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Gly		
			30					35						40			
AAG	AGA	TGC	CTA	CTG	CGT	GCT	ATT	TCT	CAG	TCT	CTA	AGA	GGA	GTG	ATC		254
Lys	Arg	Cys	Leu	Leu	Arg	Ala	Ile	Ser	Gln	Ser	Leu	Arg	Gly	Val	Ile		
			45				50					55					
AAG	GTA	TGT	GGT	CAC	ACT	TGAATCACGC	AGGTGTGTGA	AATCTCATTG									302
Lys	Val	Cys	Gly	His	Thr												
			60														
TGAACAAATA	AAAATCATGA	AAGGAAAACT	CTATGTTTGA	AATATCTTAT	GGGTCTCCT			362									
GTAAAGCTCT	TCACTCCATA	AGGTGAAATA	GACCTGAAAT	ATATATAGAT	TATTTT			417									

Figure 13 F

## CODING SEGMENT G:

Glu	Ile	Thr	Thr	Gly	Met	Pro	Ala	Ser	Thr	Glu	Thr	Ala	Tyr	Val	Ser	
AG	ATC	ACC	ACT	GGC	ATG	CCA	GCC	TCA	ACT	GAG	ACA	GCG	TAT	GTG	TCT	47
AG	ATC	ATC	ACT	GGT	ATG	CCA	GCC	TCA	ACT	GAA	GGA	GCA	TAT	GTG	TCT	
			I								G					
Ser	Glu	Ser	Pro	Ile	Arg	Ile	Ser	Val	Ser	Thr	Glu	Gly	Thr	Asn	Thr	
TCA	GAG	TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCA	ACA	GAA	GGA	ACA	AAT	ACT	95
TCA	GAG	TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCC	ACA	GAA	GGA	GCA	AAT	ACT	
													A			
Ser	Ser	Ser														
TCT	TCA	T														102
TCT	TCA	T														

Figure 13 G

## CODING SEGMENT C:

Thr	Ser	Thr	Ser	Thr	Ala	Gly	Thr	Ser	His	Leu	Val	Lys	Cys	Ala		
CC	ACA	TCC	ACA	TCT	ACA	GCT	GGG	ACA	AGC	CAT	CTT	GTC	AAG	TGT	GCA	47
CT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	AGC	CAT	CTT	GTA	AAA	TGT	GCG	
						T										
Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	Gly	Gly	Glu	Cys	Phe	Met	Val	
GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	GGA	GGC	GAG	TGC	TTC	ATG	GTG	95
GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	GGA	GGG	GAG	TGC	TTC	ATG	GTG	
Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	Leu	Cys						
AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	TTG	TGC						128
AAA	GAC	CTT	TCA	AAC	CCC	TCG	AGA	TAC	TTG	TGC						

**Figure 13 H**

## CODING SEGMENT C/D:

Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	Val	Pro	
AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	GTG	CCC	48
AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCA	AGA	TGT	ACT	GAG	AAT	GTG	CCC	

Met	Lys	Val	Gln	Thr	Gln	Glu										
ATG	AAA	GTC	CAA	ACC	CAA	GAA										69
ATG	AAA	GTC	CAA	AAC	CAA	GAA										
				N												

**Figure 13 I**

## CODING SEGMENT C/D':

Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	
AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	48
AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	

Ala	Ser	Phe	Tyr													
GCC	AGC	TTC	TAC													60
GCC	AGC	TTC	TAC													

**Figure 13 J**

## CODING SEGMENT D:

Ser	Thr	Ser	Thr	Pro	Phe	Leu	Ser	Leu	Pro	Glu	*					
AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA	TAG					36
AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA	TAG					

**Figure 13 K**

## CODING SEGMENT D':

Lys	His	Leu	Gly	Ile	Glu	Phe	Met	Glu								
AAG	CAT	CTT	GGG	ATT	GAA	TTT	ATG	GAG								27

Figure 13 L

## CODING SEGMENT H:

Lys	Ala	Glu	Glu	Leu	Tyr	Gln	Lys	Arg	Val	Leu	Thr	Ile	Thr	Gly	Ile	
AAA	GCG	GAG	GAG	CTC	TAC	CAG	AAG	AGA	GTG	CTC	ACC	ATT	ACC	GGC	ATT	48
AAA	GCG	GAG	GAG	CTG	TAC	CAG	AAG	AGA	GTG	CTG	ACC	ATA	ACC	GGC	ATC	
Cys	Ile	Ala	Leu	Leu	Val	Val	Gly	Ile	Met	Cys	Val	Val	Val	Tyr	Cys	
TGC	ATC	GCG	CTG	CTC	GTG	GTT	GGC	ATC	ATG	TGT	GTG	GTG	GTC	TAC	TGC	96
TGC	ATC	GCC	CTC	CTT	GTG	GTC	GGC	ATC	ATG	TGT	GTG	GTG	GCC	TAC	TGC	
																A
Lys	Thr	Lys	Lys	Gln	Arg	Lys	Lys	Leu	His	Asp	Arg	Leu	Arg	Gln	Ser	
AAA	ACC	AAG	AAA	CAA	CGG	AAA	AAG	CTT	CAT	GAC	CGG	CTT	CGG	CAG	AGC	144
AAA	ACC	AAG	AAA	CAG	CGG	AAA	AAG	CTG	CAT	GAC	CGT	CTT	CGG	CAG	AGC	
Leu	Arg	Ser	Glu	Arg	Asn	Thr	Met	Met	Asn	Val	Ala	Asn	Gly	Pro	His	
CTT	CGG	TCT	GAA	AGA	AAC	ACC	ATG	ATG	AAC	GTA	GCC	AAC	GGG	CCC	CAC	192
CTT	CGG	TCT	GAA	CGA	AAC	AAT	ATG	ATG	AAC	ATT	GCC	AAT	GGG	CCT	CAC	
						N				I						
His	Pro	Asn	Pro	Pro	Pro	Glu	Asn	Val	Gln	Leu	Val	Asn	Gln	Tyr	Val	
CAC	CCC	AAT	CCG	CCC	CCC	GAG	AAC	GTG	CAG	CTG	GTG	AAT	CAA	TAC	GTA	240
CAT	CCT	AAC	CCA	CCC	CCC	GAG	AAT	GTC	CAG	CTG	GTG	AAT	CAA	TAC	GTA	
Ser	Lys	Asn	Val	Ile	Ser	Ser	Glu	His	Ile	Val	Glu	Arg	Glu	Ala	Glu	
TCT	AAA	AAT	GTC	ATC	TCT	AGC	GAG	CAT	ATT	GTT	GAG	AGA	GAG	GCG	GAG	288
TCT	AAA	AAC	GTC	ATC	TCC	AGT	GAG	CAT	ATT	GTT	GAG	AGA	GAA	GCA	GAG	

Figure 13 L'

Ser	Ser	Phe	Ser	Thr	Ser	His	Tyr	Thr	Ser	Thr	Ala	His	His	Ser	Thr	
AGC	TCT	TTT	TCC	ACC	AGT	CAC	TAC	ACT	TCG	ACA	GCT	CAT	CAT	TCC	ACT	336
ACA	TCC	TTT	TCC	ACC	AGT	CAC	TAT	ACT	TCC	ACA	GCC	CAT	CAC	TCC	ACT	
T																
Thr	Val	Thr	Gln	Thr	Pro	Ser	His	Ser	Trp	Ser	Asn	Gly	His	Thr	Glu	
ACT	GTC	ACT	CAG	ACT	CCC	AGT	CAC	AGC	TGG	AGC	AAT	GGA	CAC	ACT	GAA	384
ACT	GTC	ACC	CAG	ACT	CCT	AGC	CAC	AGC	TGG	AGC	AAC	GGA	CAC	ACT	GAA	
Ser	Ile	Ile	Ser	Glu	Ser	His	Ser	Val	Ile	Val	Met	Ser	Ser	Val	Glu	
AGC	ATC	ATT	TCG	GAA	AGC	CAC	TCT	GTC	ATC	GTG	ATG	TCA	TCC	GTA	GAA	432
AGC	ATC	CTT	TCC	GAA	AGC	CAC	TCT	GTA	ATC	GTG	ATG	TCA	TCC	GTA	GAA	
Asn	Ser	Arg	His	Ser	Ser	Pro	Thr	Gly	Gly	Pro	Arg	Gly	Arg	Leu	Asn	
AAC	AGT	AGG	CAC	AGC	AGC	CCG	ACT	GGG	GGC	CCG	AGA	GGA	CGT	CTC	AAT	480
AAC	AGT	AGG	CAC	AGC	AGC	CCA	ACT	GGG	GGC	CCA	AGA	GGA	CGT	CTT	AAT	
Gly	Leu	Gly	Gly	Pro	Arg	Glu	Cys	Asn	Ser	Phe	Leu	Arg	His	Ala	Arg	
GGC	TTG	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	528
GGC	ACA	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	
T																
Glu	Thr	Pro	Asp	Ser	Tyr	Arg	Asp	Ser	Pro	His	Ser	Glu	Arg			
GAA	ACC	CCT	GAC	TCC	TAC	CGA	GAC	TCT	CCT	CAT	AGT	GAA	AG			569
GAA	ACC	CCT	GAT	TCC	TAC	CGA	GAC	TCT	CCT	CAT	AGT	GAA	AG			



## Figure 13 M

## CODING SEGMENT K:

A	CAT	AAC	CTT	ATA	GCT	GAG	CTA	AGG	AGA	AAC	AAG	GCC	CAC	AGA	TCC	46
	His	Asn	Leu	Ile	Ala	Glu	Leu	Arg	Arg	Asn	Lys	Ala	His	Arg	Ser	
	1				5					10				15		
AAA	TGC	ATG	CAG	ATC	CAG	CTT	TCC	GCA	ACT	CAT	CTT	AGA	GCT	TCT	TCC	94
Lys	Cys	Met	Gln	Ile	Gln	Leu	Ser	Ala	Thr	His	Leu	Arg	Ala	Ser	Ser	
			20						25				30			
ATT	CCC	CAT	TGG	GCT	TCA	TTC	TCT	AAG	ACC	CCT	TGG	CCT	TTA	GGA	AG	141
Ile	Pro	His	Trp	Ala	Ser	Phe	Ser	Lys	Thr	Pro	Trp	Pro	Leu	Gly	Arg	
			35					40					45			

Figure 13 N

## CODING SEGMENT L:

Tyr	Val	Ser	Ala	Met	Thr	Thr	Pro	Ala	Arg	Met	Ser	Pro	Val	Asp			46
G	TAT	GTA	TCA	GCA	ATG	ACC	ACC	CCG	GCT	CGT	ATG	TCA	CCT	GTA	GAT		
G	TAT	GTG	TCA	GCC	ATG	ACC	ACC	CCG	GCT	CGT	ATG	TCA	CCT	GTA	GAT		
Phe	His	Thr	Pro	Ser	Ser	Pro	Lys	Ser	Pro	Pro	Ser	Glu	Met	Ser	Pro		94
TTC	CAC	ACG	CCA	AGC	TCC	CCC	AAG	TCA	CCC	CCT	TCG	GAA	ATG	TCC	CCG		
TTC	CAC	ACG	CCA	AGC	TCC	CCC	AAA	TCG	CCC	CCT	TCG	GAA	ATG	TCT	CCA		
Pro	Val	Ser	Ser	Thr	Thr	Val	Ser	Met	Pro	Ser	Met	Ala	Val	Ser	Pro		142
CCC	GTG	TCC	AGC	ACG	ACG	GTC	TCC	ATG	CCC	TCC	ATG	GCG	GTC	AGT	CCC		
CCC	GTG	TCC	AGC	ATG	ACG	GTG	TCC	ATG	CCT	TCC	ATG	GCG	GTC	AGC	CCC		
				M													
Phe	Val	Glu	Glu	Glu	Arg	Pro	Leu	Leu	Leu	Val	Thr	Pro	Pro	Arg	Leu		190
TTC	GTG	GAA	GAG	GAG	AGA	CCC	CTG	CTC	CTT	GTG	ACG	CCA	CCA	CGG	CTG		
TTC	ATG	GAA	GAA	GAG	AGA	CCT	CTA	CTT	CTC	GTG	ACA	CCA	CCA	AGG	CTG		
				N													
Arg	Glu	Lys	-	Tyr	Asp	His	His	Ala	Gln	Gln	Phe	Asn	Ser	Phe	His		238
CGG	GAG	AAG	...	TAT	GAC	CAC	CAC	GCC	CAG	CAA	TTC	AAC	TCG	TTC	CAC		
CGG	GAG	AAG	AAG	TTT	GAC	CAT	CAC	CCT	CAG	CAG	TTC	AGC	TCC	TTC	CAC		
			K	F				P									
Cys	Asn	Pro	Ala	His	Glu	Ser	Asn	Ser	Leu	Pro	Pro	Ser	Pro	Leu	Arg		286
TGC	AAC	CCC	GCG	CAT	GAG	AGC	AAC	AGC	CTG	CCC	CCC	AGC	CCC	TTG	AGG		
CAC	AAC	CCC	GCG	CAT	GAC	AGT	AAC	AGC	CTC	CCT	GCT	AGC	CCC	TTG	AGG		
N				D						A							

Figure 13 N'

Ile	Val	Glu	Asp	Glu	Glu	Tyr	Glu	Thr	Thr	Gln	Glu	Tyr	Glu	Pro	Ala		
ATA	GTG	GAG	GAT	GAG	GAA	TAT	GAA	ACG	ACC	CAG	GAG	TAC	GAA	CCA	GCT		334
ATA	GTG	GAG	GAT	GAG	GAG	TAT	GAA	ACG	ACC	CAA	GAG	TAC	GAG	CCA	GCC		
Gln	Glu	Pro	Val	Lys	Lys	Leu	Thr	Asn	Ser	Ser	Arg	Arg	Ala	Lys	Arg		
CAA	GAG	CCG	GTT	AAG	AAA	CTC	ACC	AAC	AGC	AGC	CGG	CGG	GCC	AAA	AGA		382
CAA	GAG	CCT	GTT	AAG	AAA	CTC	GCC	AA.	..T	AGC	CGG	CGG	GCC	AAA	AGA		
							A										
Thr	Lys	Pro	Asn	Gly	His	Ile	Ala	His	Arg	Leu	Glu	Met	Asp	Asn	Asn		
ACC	AAG	CCC	AAT	GGT	CAC	ATT	GCC	CAC	AGG	TTG	GAA	ATG	GAC	AAC	AAC		430
ACC	AAG	CCC	AAT	GGC	CAC	ATT	GCT	AAC	AGA	TTG	GAA	GTG	GAC	AGC	AAC		
							N					V		S			
Thr	Gly	Ala	Asp	Ser	Ser	Asn	Ser	Glu	Ser	Glu	Thr	Glu	Asp	Glu	Arg		
ACA	GGC	GCT	GAC	AGC	AGT	AAC	TCA	GAG	AGC	GAA	ACA	GAG	GAT	GAA	AGA		478
ACA	AGC	TCC	CAG	AGC	AGT	AAC	TCA	GAG	AGT	GAA	ACA	GAA	GAT	GAA	AGA		
	S	S	Q														

Figure 13 N''

Val	Gly	Glu	Asp	Thr	Pro	Phe	Leu	Ala	Ile	Gln	Asn	Pro	Leu	Ala	Ala	
GTA	GGA	GAA	GAT	ACG	CCT	TTC	CTG	GCC	ATA	CAG	AAC	CCC	CTG	GCA	GCC	526
GTA	GGT	GAA	GAT	ACG	CCT	TTC	CTG	GGC	ATA	CAG	AAC	CCC	CTG	GCA	GCC	
								G								
Ser	Leu	Glu	Ala	Ala	Pro	Ala	Phe	Arg	Leu	Val	Asp	Ser	Arg	Thr	Asn	
AGT	CTC	GAG	GCG	GCC	CCT	GCC	TTC	CGC	CTG	GTC	GAC	AGC	AGG	ACT	AAC	574
AGT	CTT	GAG	GCA	ACA	CCT	GCC	TTC	CGC	CTG	GCT	GAC	AGC	AGG	ACT	AAC	
				T						A						
Pro	Thr	Gly	Gly	Phe	Ser	Pro	Gln	Glu	Glu	Leu	Gln	Ala	Arg	Leu	Ser	
CCA	ACA	GGC	GGC	TTC	TCT	CCG	CAG	GAA	GAA	TTG	CAG	GCC	AGG	CTC	TCC	622
CCA	GCA	GGC	CGC	TTC	TCG	ACA	CAG	GAA	GAA	ATC	CAG	GCC	AGG	CTG	TCT	
	A		R			T				I						
Gly	Val	Ile	Ala	Asn	Gln	Asp	Pro	Ile	Ala	Val	*					
GGT	GTA	ATC	GCT	AAC	CAA	GAC	CCT	ATC	GCT	GTC	TAA	AAC	CGA	AAT	ACA	672
AGT	GTA	ATT	GCT	AAC	CAA	GAC	CCT	ATT	GCT	GTA	TAA	AAC	CTA	AAT	AAA	
S																
CCC	ATA	GAT	TCA	CCT	GTA	AAA	CTT	TAT	TTT	ATA	TAA	TAA	AGT	ATT	CCA	718
CAC	ATA	GAT	TCA	CCT	GTA	AAA	CTT	TAT	TTT	ATA	TAA	TAA	AGT	ATT	CCA	
CCT	TAA	ATT	AAA	CAA												733
CCT	TAA	ATT	AAA	CAA												

Figure 13 O

## HUMAN CODING SEGMENT E:

ATG AGA TGG CGA CGC GCC CCG CGC CGC TCC GGG CGT CCC GGC CCC CGG	48
Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg	
1 5 10 15	
GCC CAG CGC CCC GGC TCC GCC GCC CGC TCG TCG CCG CCG CTG CCG CTG	96
Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu	
20 25 30	
CTG CCA CTA CTG CTG CTG CTG GGG ACC GCG GCC CTG GCG CCG GGG GCG	144
Leu Pro Leu Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala	
35 40 45	
GCG GCC GGC AAC GAG GCG GCT CCC GCG GGG GCC TCG GTG TGC TAC TCG	192
Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser	
50 55 60	
TCC CCG CCC AGC GTG GGA TCG GTG CAG GAG CTA GCT CAG CGC GCC GCG	240
Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala	
65 70 75 80	
GTG GTG ATC GAG GGA AAG GTG CAC CCG CAG CGG CGG CAG CAG GGG GCA	288
Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala	
85 90 95	
CTC GAC AGG AAG GCG GCG GCG GCG GCG GGC GAG GCA GGG GCG TGG GGC	336
Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly	
100 105 110	
GGC GAT CGC GAG CCG CCA GCC GCG GGC CCA CGG GCG CTG GGG CCG CCC	384
Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro	
115 120 125	
GCC GAG GAG CCG CTG CTC GCC GCC AAC GGG ACC GTG CCC TCT TGG CCC	432
Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro	
130 135 140	
ACC GCC CCG GTG CCC AGC GCC GGC GAG CCC GGG GAG GAG GCG CCC TAT	480
Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr	
145 150 155 160	
CTG GTG AAG GTG CAC CAG GTG TGG GCG GTG AAA GCC GGG GGC TTG AAG	528
Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys	
165 170 175	
AAG GAC TCG CTG CTC ACC GTG CGC CTG GGG ACC TGG GGC CAC CCC GCC	576
Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala	
180 185 190	
TTC CCC TCC TGC GGG AGG CTC AAG GAG GAC AGC AGG TAC ATC TTC TTC	624
Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe	
195 200 205	
ATG GAG CCC GAC GCC AAC AGC ACC AGC CGC GCG CCG GCC GCC TTC CGA	672
Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg	
210 215 220	
GCC TCT TTC CCC CCT CTG GAG ACG GGC CGG AAC CTC AAG AAG GAG GTC	720
Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val	
225 230 235 240	
AGC CGG GTG CTG TGC AAG CGG TGC G	745
Ser Arg Val Leu Cys Lys Arg Cys	
245	

Figure 14 A

AGTTTCCCCC	CCCAACTTGT	CGGAACTCTG	GGCTCGCGCG	CAGGGCAGGA	GCGGAGCGGC	60
GGCGGCTGCC	CAGGCGATGC	GAGCGCGGGC	CGGACGGTAA	TCGCCCTCTCC	CTCCTCGGGC	120
TGCGAGCGCG	CCGGACCGAG	GCAGCGACAG	GAGCGGACCG	CGGCGGGAAC	CGAGGACTCC	180
CCAGCGGCGC	GCCAGCAGGA	GCCACCCCGC	GAGCGTGCGA	CCGGGACGGA	GCGCCCGCCA	240
GTCCCAGGTG	GCCCGGACCG	CACGTTGCGT	CCCCGCGCTC	CCCGCCGGCG	ACAGGAGACG	300
CTCCCCCCCA	CGCCGCGCGC	GCCTCGGCCC	GGTCGCTGGC	CCGCCCTCCAC	TCCGGGGACA	360
AACTTTTCCC	GAAGCCGATC	CCAGCCCTCG	GACCCAAACT	TGTCGCGCGT	CGCCTTCGCC	420
GGGAGCCGTC	CGCGCAGAGC	GTGCACTTCT	CGGGCGAG	ATG TCG GAG CGC AGA		475
				Met Ser Glu Arg Arg		
				1 5		
GAA GGC AAA GGC AAG GGG AAG GGC GGC AAG AAG GAC CGA GGC TCC GGG						523
Glu Gly Lys Gly Lys Gly Lys Gly Gly Lys Lys Asp Arg Gly Ser Gly						
	10		15		20	
AAG AAG CCC GTG CCC GCG GCT GGC GGC CCG AGC CCA GCC TTG CCT CCC						571
Lys Lys Pro Val Pro Ala Ala Gly Gly Pro Ser Pro Ala Leu Pro Pro						
	25		30		35	
CGC TTG AAA GAG ATG AAG ATG CAG GAG TCT GTG GCA GGT TCC AAA CTA						619
Arg Leu Lys Glu Met Lys Ser Gln Glu Ser Val Ala Gly Ser Lys Leu						
	40		45		50	
GTG CTT CGG TGC GAG ACC AGT TCT GAA TAC TCC TCT CTC AAG TTC AAG						667
Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu Lys Phe Lys						
	55		60		65	
TGG TTC AAG AAT GGG AGT GAA TTA AGC CGA AAG AAC AAA CCA CAA AAC						715
Trp Phe Lys Asn Gly Ser Glu Leu Ser Arg Lys Asn Lys Pro Gln Asn						
	70		75		80	85
ATC AAG ATA CAG AAA AGG CCG GGG AAG TCA GAA CTT CGC ATT AGC AAA						763
Ile Lys Ile Gln Lys Arg Pro Gly Lys Ser Glu Leu Arg Ile Ser Lys						
	90		95		100	
GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC AGC AAA						811
Ala Ser Leu Ala Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys						
	105		110		115	

Figure 14 B

CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG TCA AAC Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu Ser Asn 120 125 130	859
GAG ATC ACC ACT GGC ATG CCA GCC TCA ACT GAG ACA GCG TAT GTG TCT Glu Ile Thr Thr Gly Met Pro Ala Ser Thr Glu Thr Ala Tyr Val Ser 135 140 145	907
TCA GAG TCT CCC ATT AGA ATA TCA GTA TCA ACA GAA GGA ACA AAT ACT Ser Glu Ser Pro Ile Arg Ile Ser Val Ser Thr Glu Gly Thr Asn Thr 150 155 160 165	955
TCT TCA TCC ACA TCC ACA TCT ACA GCT GGG ACA AGC CAT CTT GTC AAG Ser Ser Ser Thr Ser Thr Ser Thr Ala Gly Thr Ser His Leu Val Lys 170 175 180	1003
TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGC GAG TGC TTC Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys Phe 185 190 195	1051
ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC TTG TGC AAG TGC CCA Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys Pro 200 205 210	1099
AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe 215 220 225	1147
TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA TAGGCGCATG Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu 230 235 240	1193
CTCAGTCGGT GCCGCTTTCT TGTTGCCGCA TCTCCCTCA GATTCAACCT AGAGCTAGAT	1253
GCGTTTTACC AGGTCTAACA TTGACTGCCT CTGCCTGTCG CATGAGAACA TTAACACAAG	1313
CGATTGTATG ACTTCCTCTG TCCGTGACTA GTGGGCTCTG AGCTACTCGT AGGTGCGTAA	1373
GGCTCCAGTG TTTCTGAAAT TGATCTTGAA TTACTGTGAT ACGACATGAT AGTCCCTCTC	1433
ACCCAGTGCA ATGACAATAA AGGCCTTGAA AAGTCTCACT TTTATTGAGA AAATAAAAAT	1493
CGTTCCACGG GACAGTCCCT CTTCTTTATA AAATGACCCT ATCCTTGAAA AGGAGGTGTG	1553
TTAAGTTGTA ACCAGTACAC ACTTGAAATG ATGGTAAGTT CGCTTCGGTT CAGAATGTGT	1613
TCTTTCTGAC AAATAAACAG AATAAAAAAA AAAAAAAAAA A	1654

Figure 15 A

CAT	CAN	GTG	TGG	GCG	GCG	AAA	GCC	GGG	GGC	TTG	AAG	AAG	GAC	TCG	CTG	48
His	Gln	Val	Trp	Ala	Ala	Lys	Ala	Gly	Gly	Leu	Lys	Lys	Asp	Ser	Leu	
1				5				10					15			
CTC	ACC	GTG	CGC	CTG	GGC	GCC	TGG	GGC	CAC	CCC	GCC	TTC	CCC	TCC	TGC	96
Leu	Thr	Val	Arg	Leu	Gly	Ala	Trp	Gly	His	Pro	Ala	Phe	Pro	Ser	Cys	
			20					25					30			
GGG	CGC	CTC	AAG	GAG	GAC	AGC	AGG	TAC	ATC	TTC	TTC	ATG	GAG	CCC	GAG	144
Gly	Arg	Leu	Lys	Glu	Asp	Ser	Arg	Tyr	Ile	Phe	Phe	Met	Glu	Pro	Glu	
		35					40					45				
GCC	AAC	AGC	AGC	GGC	GGG	CCC	GGC	CGC	CTT	CCG	AGC	CTC	CTT	CCC	CCC	192
Ala	Asn	Ser	Ser	Gly	Gly	Pro	Gly	Arg	Leu	Pro	Ser	Leu	Leu	Pro	Pro	
	50					55					60					
TCT	CGA	GAC	GGG	CCG	GAA	CCT	CAA	GAA	GGA	GGT	CAG	CCG	GGT	GCT	GTG	240
Ser	Arg	Asp	Gly	Pro	Glu	Pro	Gln	Glu	Gly	Gln	Pro	Gly	Ala	Val		
65				70				75						80		
CAA	CGG	TGC	GCC	TTG	CCT	CCC	CGC	TTG	AAA	GAG	ATG	AAG	AGT	CAG	GAG	288
Gln	Arg	Cys	Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu	
			85					90						95		
TCT	GTG	GCA	GGT	TCC	AAA	CTA	GTG	CTT	CGG	TGC	GAG	ACC	AGT	TCT	GAA	336
Ser	Val	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu	
		100						105					110			
TAC	TCC	TCT	CTC	AAG	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AGT	GAA	TTA	AGC	384
Tyr	Ser	Ser	Leu	Lys	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Ser	Glu	Leu	Ser	
		115					120					125				
CGA	AAG	AAC	AAA	CCA	GAA	AAC	ATC	AAG	ATA	CAG	AAA	AGG	CCG	GGG	AAG	432
Arg	Lys	Asn	Lys	Pro	Glu	Asn	Ile	Lys	Ile	Gln	Lys	Arg	Pro	Gly	Lys	
	130					135					140					
TCA	GAA	CTT	CGC	ATT	AGC	AAA	GCG	TCA	CTG	GCT	GAT	TCT	GGA	GAA	TAT	480
Ser	Glu	Leu	Arg	Ile	Ser	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	
145				150					155					160		
ATG	TGC	AAA	GTG	ATC	AGC	AAA	CTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAC	528
Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	
				165				170					175			



## Figure 15 B

ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG ACA Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly Thr 180 185 190	576
AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn 195 200 205	624
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr 210 215 220	672
TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG AAT Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn 225 230 235 240	720
GTG CCC ATG AAA GTC CAA ACC CAA GAA AAG TGC CCA AAT GAG TTT ACT Val Pro Met Lys Val Gln Thr Gln Glu Lys Cys Pro Asn Glu Phe Thr 245 250 255	768
GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC TTC TAC AGT ACG TCC Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser Phe Tyr Ser Thr Ser 260 265 270	816
ACT CCC TTT CTG TCT CTG CCT GAA TAGCGCATCT CAGTCGGTGC CGCTTTCTTG Thr Pro Phe Leu Ser Leu Pro Glu 275 280	870
TTGCCGCATC TCCCCTCAGA TTCCNCCTAG AGCTAGATGC GTTTTACCAG GTCTAACATT	930
GACTGCCTCT GCCTGTCGCA TGAGAACATT AACACAAGCG ATTGTATGAC TTCCTCTGTC	990
CGTGACTAGT GGGCTCTGAG CTACTCGTAG GTGCGTAAGG CTCCAGTGTT TCTGAAATTG	1050
ATCTTGAATT ACTGTGATAC GACATGATAG TCCCTCTCAC CCAGTGCAAT GACAATAAAG	1110
GCCTTGAAAA GTCAAAAAA AAAAAAAAAA	1140

Figure 16 A

G AAG TCA GAA CTT CGC ATT AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA	49
Lys Ser Glu Leu Arg Ile Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu	
1 5 10 15	
TAT ATG TGC AAA GTG ATC AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC	97
Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala	
20 25 30	
AAC ATC ACC ATT GTG GAG TCA AAC GCC ACA TCC ACA TCT ACA GCT GGG	145
Asn Ile Thr Ile Val Glu Ser Asn Ala Thr Ser Thr Ser Thr Ala Gly	
35 40 45	
ACA AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG	193
Thr Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val	
50 55 60	
AAT GGA GGC GAC TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA	241
Asn Gly Gly Asp Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg	
65 70 75 80	
TAC TTG TGC AAG TGC CAA CCT GGA TTC ACT GGA GCG AGA TGT ACT GAG	289
Tyr Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu	
85 90 95	
AAT GTG CCC ATG AAA GTC CAA ACC CAA GAA AAA GCG GAG GAG CTC TAC	337
Asn Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr	
100 105 110	
CAG AAG AGA GTG CTC ACC ATT ACC GGC ATT TGC ATC GCG CTG CTC GTG	385
Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile Ala Leu Leu Val	
115 120 125	
GTT GGC ATC ATG TGT GTG GTG GTC TAC TGC AAA ACC AAG AAA CAA CGG	433
Val Gly Ile Met Cys Val Val Val Tyr Cys Lys Thr Lys Lys Gln Arg	
130 135 140	
AAA AAG CTT CAT GAC CGG CTT CGG CAG AGC CTT CGG TCT GAA AGA AAC	481
Lys Lys Leu His Asp Arg Leu Arg Gln Ser Leu Arg Ser Glu Arg Asn	
145 150 155 160	
ACC ATG ATG AAC GTA GCC AAC GGG CCC CAC CAC CCC AAT CCG CCC CCC	529
Thr Met Met Asn Val Ala Asn Gly Pro His His Pro Asn Pro Pro Pro	
165 170 175	
GAG AAC GTG CAG CTG GTG AAT CAA TAC GTA TCT AAA AAT GTC ATC TCT	577
Glu Asn Val Gln Leu Val Asn Gln Tyr Val Ser Lys Asn Val Ile Ser	
180 185 190	

Figure 16 B

AGC	GAG	CAT	ATT	GTT	GAG	AGA	GAG	GCG	GAG	AGC	TCT	TTT	TCC	ACC	AGT	625
Ser	Glu	His	Ile	Val	Glu	Arg	Glu	Ala	Glu	Ser	Ser	Phe	Ser	Thr	Ser	
		195					200					205				
CAC	TAC	ACT	TCG	ACA	GCT	CAT	CAT	TCC	ACT	ACT	GTC	ACT	CAG	ACT	CCC	673
His	Tyr	Thr	Ser	Thr	Ala	His	His	Ser	Thr	Thr	Val	Thr	Gln	Thr	Pro	
	210					215					220					
AGT	CAC	AGC	TGG	AGC	AAT	GGA	CAC	ACT	GAA	AGC	ATC	ATT	TCG	GAA	AGC	721
Ser	His	Ser	Trp	Ser	Asn	Gly	His	Thr	Glu	Ser	Ile	Ile	Ser	Glu	Ser	
225					230				235					240		
CAC	TCT	GTC	ATC	GTG	ATG	TCA	TCC	GTA	GAA	AAC	AGT	AGG	CAC	AGC	AGC	769
His	Ser	Val	Ile	Val	Met	Ser	Ser	Val	Glu	Asn	Ser	Arg	His	Ser	Ser	
				245					250					255		
CCG	ACT	GGG	GGC	CCG	AGA	GGA	CGT	CTC	AAT	GGC	TTG	GGA	GGC	CCT	CGT	817
Pro	Thr	Gly	Gly	Pro	Arg	Gly	Arg	Leu	Asn	Gly	Leu	Gly	Gly	Pro	Arg	
			260					265					270			
GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	GAA	ACC	CCT	GAC	TCC	TAC	865
Glu	Cys	Asn	Ser	Phe	Leu	Arg	His	Ala	Arg	Glu	Thr	Pro	Asp	Ser	Tyr	
		275					280					285				
CGA	GAC	TCT	CCT	CAT	AGT	GAA	AGA	CAT	AAC	CTT	ATA	GCT	GAG	CTA	AGG	913
Arg	Asp	Ser	Pro	His	Ser	Glu	Arg	His	Asn	Leu	Ile	Ala	Glu	Leu	Arg	
	290					295					300					
AGA	AAC	AAG	GCC	CAC	AGA	TCC	AAA	TGC	ATG	CAG	ATC	CAG	CTT	TCC	GCA	961
Arg	Asn	Lys	Ala	His	Arg	Ser	Lys	Cys	Met	Gln	Ile	Gln	Leu	Ser	Ala	
305					310					315					320	
ACT	CAT	CTT	AGA	GCT	TCT	TCC	ATT	CCC	CAT	TGG	GCT	TCA	TTC	TCT	AAG	1009
Thr	His	Leu	Arg	Ala	Ser	Ser	Ile	Pro	His	Trp	Ala	Ser	Phe	Ser	Lys	
				325				330						335		
ACC	CCT	TGG	CCT	TTA	GGA	AGG	TAT	GTA	TCA	GCA	ATG	ACC	ACC	CCG	GCT	1057
Thr	Pro	Trp	Pro	Leu	Gly	Arg	Tyr	Val	Ser	Ala	Met	Thr	Thr	Pro	Ala	
			340					345					350			
CGT	ATG	TCA	CCT	GTA	GAT	TTC	CAC	ACG	CCA	AGC	TCC	CCC	AAG	TCA	CCC	1105
Arg	Met	Ser	Pro	Val	Asp	Phe	His	Thr	Pro	Ser	Ser	Pro	Lys	Ser	Pro	
		355					360					365				
CCT	TCG	GAA	ATG	TCC	CCG	CCC	GTG	TCC	AGC	ACG	ACG	GTC	TCC	ATG	CCC	1153
Pro	Ser	Glu	Met	Ser	Pro	Pro	Val	Ser	Ser	Thr	Thr	Val	Ser	Met	Pro	
	370					375						380				

Figure 16 C

TCC ATG GCG GTC AGT CCC TTC GTG GAA GAG GAG AGA CCC CTG CTC CTT Ser Met Ala Val Ser Pro Phe Val Glu Glu Glu Arg Pro Leu Leu Leu 385 390 395 400	1201
GTG ACG CCA CCA CGG CTG CGG GAG AAG TAT GAC CAC CAC GCC CAG CAA Val Thr Pro Pro Arg Leu Arg Glu Lys Tyr Asp His His Ala Gln Gln 405 410 415	1249
TTC AAC TCG TTC CAC TGC AAC CCC GCG CAT GAG AGC AAC AGC CTG CCC Phe Asn Ser Phe His Cys Asn Pro Ala His Glu Ser Asn Ser Leu Pro 420 425 430	1297
CCC AGC CCC TTG AGG ATA GTG GAG GAT GAG GAA TAT GAA ACG ACC CAG Pro Ser Pro Leu Arg Ile Val Glu Asp Glu Glu Tyr Glu Thr Thr Gln 435 440 445	1345
GAG TAC GAA CCA GCT CAA GAG CCG GTT AAG AAA CTC ACC AAC AGC AGC Glu Tyr Glu Pro Ala Gln Glu Pro Val Lys Lys Leu Thr Asn Ser Ser 450 455 460	1393
CGG CGG GCC AAA AGA ACC AAG CCC AAT GGT CAC ATT GCC CAC AGG TTG Arg Arg Ala Lys Arg Thr Lys Pro Asn Gly His Ile Ala His Arg Leu 465 470 475 480	1441
GAA ATG GAC AAC AAC ACA GGC GCT GAC AGC AGT AAC TCA GAG AGC GAA Glu Met Asp Asn Asn Thr Gly Ala Asp Ser Ser Asn Ser Glu Ser Glu 485 490 495	1489
ACA GAG GAT GAA AGA GTA GGA GAA GAT ACG CCT TTC CTG GCC ATA CAG Thr Glu Asp Glu Arg Val Gly Glu Asp Thr Pro Phe Leu Ala Ile Gln 500 505 510	1537
AAC CCC CTG GCA GCC AGT CTC GAG GCG GCC CCT GCC TTC CGC CTG GTC Asn Pro Leu Ala Ala Ser Leu Glu Ala Ala Pro Ala Phe Arg Leu Val 515 520 525	1585
GAC AGC AGG ACT AAC CCA ACA GGC GGC TTC TCT CCG CAG GAA GAA TTG Asp Ser Arg Thr Asn Pro Thr Gly Gly Phe Ser Pro Gln Glu Glu Leu 530 535 540	1633
CAG GCC AGG CTC TCC GGT GTA ATC GCT AAC CAA GAC CCT ATC GCT GTC Gln Ala Arg Leu Ser Gly Val Ile Ala Asn Gln Asp Pro Ile Ala Val 545 550 555 560	1681
TAAAACCGAA ATACACCCAT AGATTCACCT GTAAAACCTTT ATTTTATATA ATAAAGTATT	1741
CCACCTTAAA TTAAACAAAA AAA	1764

Figure 17 A

## F-B-A'

F-B-A-C-C/D-D  
 F-B-A-C-C/D-H  
 F-B-A-C-C/D-H-L  
 F-B-A-C-C/D-H-K-L  
 F-B-A-C-C/D-D'-H  
 F-B-A-C-C/D-D'-H-L  
 F-B-A-C-C/D-D'-H-K-L  
 F-B-A-C-C/D'-D  
 F-B-A-C-C/D'-H  
 F-B-A-C-C/D'-H-L  
 F-B-A-C-C/D'-H-K-L  
 F-B-A-C-C/D'-D'-H  
 F-B-A-C-C/D'-D'-H-L  
 F-B-A-C-C/D'-D'-H-K-L  
 F-B-A-C-C/D-C/D'-D  
 F-B-A-C-C/D-C/D'-H  
 F-B-A-C-C/D-C/D'-H-L  
 F-B-A-C-C/D-C/D'-H-K-L  
 F-B-A-C-C/D-C/D'-D'-H  
 F-B-A-C-C/D-C/D'-D'-H-L  
 F-B-A-C-C/D-C/D'-D'-H-K-L

F-B-A-G-C-C/D-D  
 F-B-A-G-C-C/D-H  
 F-B-A-G-C-C/D-H-L  
 F-B-A-G-C-C/D-H-K-L  
 F-B-A-G-C-C/D-D'-H  
 F-B-A-G-C-C/D-D'-H-L  
 F-B-A-G-C-C/D-D'-H-K-L  
 F-B-A-G-C-C/D'-D  
 F-B-A-G-C-C/D'-H  
 F-B-A-G-C-C/D'-H-L  
 F-B-A-G-C-C/D'-H-K-L  
 F-B-A-G-C-C/D'-D'-H  
 F-B-A-G-C-C/D'-D'-H-L  
 F-B-A-G-C-C/D'-D'-H-K-L  
 F-B-A-G-C-C/D-C/D'-D  
 F-B-A-G-C-C/D-C/D'-H  
 F-B-A-G-C-C/D-C/D'-H-L  
 F-B-A-G-C-C/D-C/D'-H-K-L  
 F-B-A-G-C-C/D-C/D'-D'-H  
 F-B-A-G-C-C/D-C/D'-D'-H-L  
 F-B-A-G-C-C/D-C/D'-D'-H-K-L

## F-E-B-A'

F-E-B-A-C-C/D-D  
 F-E-B-A-C-C/D-H  
 F-E-B-A-C-C/D-H-L  
 F-E-B-A-C-C/D-H-K-L  
 F-E-B-A-C-C/D-D'-H  
 F-E-B-A-C-C/D-D'-H-L  
 F-E-B-A-C-C/D-D'-H-K-L  
 F-E-B-A-C-C/D'-D  
 F-E-B-A-C-C/D'-H  
 F-E-B-A-C-C/D'-H-L  
 F-E-B-A-C-C/D'-H-K-L  
 F-E-B-A-C-C/D'-D'-H  
 F-E-B-A-C-C/D'-D'-H-L  
 F-E-B-A-C-C/D'-D'-H-K-L  
 F-E-B-A-C-C/D-C/D'-D  
 F-E-B-A-C-C/D-C/D'-H  
 F-E-B-A-C-C/D-C/D'-H-L  
 F-E-B-A-C-C/D-C/D'-H-K-L  
 F-E-B-A-C-C/D-C/D'-D'-H  
 F-E-B-A-C-C/D-C/D'-D'-H-L  
 F-E-B-A-C-C/D-C/D'-D'-H-K-L

F-E-B-A-G-C-C/D-D  
 F-E-B-A-G-C-C/D-H  
 F-E-B-A-G-C-C/D-H-L  
 F-E-B-A-G-C-C/D-H-K-L  
 F-E-B-A-G-C-C/D-D'-H  
 F-E-B-A-G-C-C/D-D'-H-L  
 F-E-B-A-G-C-C/D-D'-H-K-L  
 F-E-B-A-G-C-C/D'-D  
 F-E-B-A-G-C-C/D'-H  
 F-E-B-A-G-C-C/D'-H-L  
 F-E-B-A-G-C-C/D'-H-K-L  
 F-E-B-A-G-C-C/D'-D'-H  
 F-E-B-A-G-C-C/D'-D'-H-L  
 F-E-B-A-G-C-C/D'-D'-H-K-L  
 F-E-B-A-G-C-C/D-C/D'-D  
 F-E-B-A-G-C-C/D-C/D'-H  
 F-E-B-A-G-C-C/D-C/D'-H-L  
 F-E-B-A-G-C-C/D-C/D'-H-K-L  
 F-E-B-A-G-C-C/D-C/D'-D'-H  
 F-E-B-A-G-C-C/D-C/D'-D'-H-L  
 F-E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 17 B

E-B-A'

E-B-A-C-C/D-D  
E-B-A-C-C/D-H  
E-B-A-C-C/D-H-L  
E-B-A-C-C/D-H-K-L  
E-B-A-C-C/D-D'-H  
E-B-A-C-C/D-D'-H-L  
E-B-A-C-C/D-D'-H-K-L  
E-B-A-C-C/D'-D  
E-B-A-C-C/D'-H  
E-B-A-C-C/D'-H-L  
E-B-A-C-C/D'-H-K-L  
E-B-A-C-C/D'-D'-H  
E-B-A-C-C/D'-D'-H-L  
E-B-A-C-C/D'-D'-H-K-L  
E-B-A-C-C/D-C/D'-D  
E-B-A-C-C/D-C/D'-H  
E-B-A-C-C/D-C/D'-H-L  
E-B-A-C-C/D-C/D'-H-K-L  
E-B-A-C-C/D-C/D'-D'-H  
E-B-A-C-C/D-C/D'-D'-H-L  
E-B-A-C-C/D-C/D'-D'-H-K-L

E-B-A-G-C-C/D-D  
E-B-A-G-C-C/D-H  
E-B-A-G-C-C/D-H-L  
E-B-A-G-C-C/D-H-K-L  
E-B-A-G-C-C/D-D'-H  
E-B-A-G-C-C/D-D'-H-L  
E-B-A-G-C-C/D-D'-H-K-L  
E-B-A-G-C-C/D'-D  
E-B-A-G-C-C/D'-H  
E-B-A-G-C-C/D'-H-L  
E-B-A-G-C-C/D'-H-K-L  
E-B-A-G-C-C/D'-D'-H  
E-B-A-G-C-C/D'-D'-H-L  
E-B-A-G-C-C/D'-D'-H-K-L  
E-B-A-G-C-C/D-C/D'-D  
E-B-A-G-C-C/D-C/D'-H  
E-B-A-G-C-C/D-C/D'-H-L  
E-B-A-G-C-C/D-C/D'-H-K-L  
E-B-A-G-C-C/D-C/D'-D'-H  
E-B-A-G-C-C/D-C/D'-D'-H-L  
E-B-A-G-C-C/D-C/D'-D'-H-K-L

Figure 18

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	144
Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	
		35					40					45				
GTA	ATG	GCC	AGC	TTC	TAC	AGT	ACG	TCC	ACT	CCC	TTT	CTG	TCT	CTG	CCT	192
Val	Met	Ala	Ser	Phe	Tyr	Ser	Thr	Ser	Thr	Pro	Phe	Leu	Ser	Leu	Pro	
	50					55					60					
GAA	TAG															198
Glu																
65																

Figure 19

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	144
Leu	Cys	Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	
		35					40					45				
GTG	CCC	ATG	AAA	GTC	CAA	ACC	CAA	GAA	AAA	GCG	GAG	GAG	CTC	TAC	TAA	192
Val	Pro	Met	Lys	Val	Gln	Thr	Gln	Glu	Lys	Ala	Glu	Glu	Leu	Tyr		
	50					55					60					



**Figure 20**

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	144
Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	
		35					40					45				
GTA	ATG	GCC	AGC	TTC	TAC	AAA	GCG	GAG	GAG	CTC	TAC	TAA				183
Val	Met	Ala	Ser	Phe	Tyr	Lys	Ala	Glu	Glu	Leu	Tyr					
	50					55					60					

**Figure 21**

AGC CAT CTT GTC AAG TGT GCA GAG AAG GAG AAA ACT TTC TGT GTG AAT	48
Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn	
1 5 10 15	
GGA GGC GAG TGC TTC ATG GTG AAA GAC CTT TCA AAT CCC TCA AGA TAC	96
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr	
20 25 30	
TTG TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC	144
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr	
35 40 45	
GTA ATG GCC AGC TTC TAC AAG CAT CTT GGG ATT GAA TTT ATG GAG AAA	192
Val Met Ala Ser Phe Tyr Lys His Leu Gly Ile Glu Phe Met Glu Lys	
50 55 60	
GCG GAG GAG CTC TAC TAA	210
Ala Glu Glu Leu Tyr	
65	

Figure 22

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	144
Leu	Cys	Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	
		35					40					45				
GTG	CCC	ATG	AAA	GTC	CAA	ACC	CAA	GAA	AAG	TGC	CCA	AAT	GAG	TTT	ACT	192
Val	Pro	Met	Lys	Val	Gln	Thr	Gln	Glu	Lys	Cys	Pro	Asn	Glu	Phe	Thr	
	50					55					60					
GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	GCC	AGC	TTC	TAC	AGT	ACG	TCC	240
Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala	Ser	Phe	Tyr	Ser	Thr	Ser	
65					70					75					80	
ACT	CCC	TTT	CTG	TCT	CTG	CCT	GAA	TAG								267
Thr	Pro	Phe	Leu	Ser	Leu	Pro	Glu									
				85												

## Figure 23

AGC	CAT	CTT	GTC	AAG	TGT	GCA	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	48
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
1				5					10					15		
GGA	GGC	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAT	CCC	TCA	AGA	TAC	96
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	
			20					25					30			
TTG	TGC	AAG	TGC	CAA	CCT	GGA	TTC	ACT	GGA	GCG	AGA	TGT	ACT	GAG	AAT	144
Leu	Cys	Lys	Cys	Gln	Pro	Gly	Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	
		35					40					45				
GTG	CCC	ATG	AAA	GTC	CAA	ACC	CAA	GAA	AAG	TGC	CCA	AAT	GAG	TTT	ACT	192
Val	Pro	Met	Lys	Val	Gln	Thr	Gln	Glu	Lys	Cys	Pro	Asn	Glu	Phe	Thr	
	50					55					60					
GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	GCC	AGC	TTC	TAC	AAA	GCG	GAG	240
Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala	Ser	Phe	Tyr	Lys	Ala	Glu	
65					70					75					80	
GAG	CTC	TAC	TAA													252
Glu	Leu	Tyr														

Figure 24 A

GGAATTCCTT TTTTTTTTTT TTTTTTCTT NNTTTTTTTT TGCCCTTATA CCTCTTCGCC	60
TTTCTGTGGT TCCATCCACT TCTTCCCCCT CCTCCTCCCA TAAACAACTC TCCTACCCCT	120
GCACCCCCAA TAAATAAATA AAAGGAGGAG GGCAAGGGGG GAGGAGGAGG AGTGGTGCTG	180
CGAGGGGAAG GAAAAGGGAG GCAGCGCGAG AAGAGCCGGG CAGAGTCCGA ACCGACAGCC	240
AGAAGCCCGC ACGCACCTCG CACC ATG AGA TGG CGA CGC GCC CCG CGC CGC	291
Met Arg Trp Arg Arg Ala Pro Arg Arg	
1 5	
TCC GGG CGT CCC GGC CCC CGG GCC CAG CGC CCC GGC TCC GCC GCC CGC	339
Ser Gly Arg Pro Gly Pro Arg Ala Gln Arg Pro Gly Ser Ala Ala Arg	
10 15 20 25	
TCG TCG CCG CCG CTG CCG CTG CTG CCA CTA CTG CTG CTG CTG GGG ACC	387
Ser Ser Pro Pro Leu Pro Leu Leu Pro Leu Leu Leu Leu Gly Thr	
Val Cys Leu Leu Thr Val	
GGF II 09	
30 35 40	
GCG GCC CTG GCG CCG GGG GCG GCG GCC GGC AAC GAG GCG GCT CCC GCG	435
Ala Ala Leu Ala Pro Gly Ala Ala Ala Gly Asn Glu Ala Ala Pro Ala	
Ala Ala Leu Pro Pro	
45 50 55	
GGG GCC TCG GTG TGC TAC TCG TCC CCG CCC AGC GTG GGA TCG GTG CAG	483
Gly Ala Ser Val Cys Tyr Ser Ser Pro Pro Ser Val Gly Ser Val Gln	
Ala Ser Pro Val Ser Val Gly Ser Val Gln	
GGF II 08	
60 65 70	
GAG CTA GCT CAG CGC   GCC GCG GTG GTG ATC GAG GGA AAG GTG CAC CCG	531
Glu Leu Ala Gln Arg   Ala Ala Val Val Ile Glu Gly Lys Val His Pro	
Glu Leu Val Gln Arg   Trp Phe Val Val Ile Glu Gly Lys	
GGF II 04	
75 80 85	

Figure 24 B

CAG CGG CGG CAG CAG GGG GCA CTC GAC AGG AAG GCG GCG GCG GCG GCG	579
Gln Arg Arg Gln Gln Gly Ala Leu Asp Arg Lys Ala Ala Ala Ala Ala	
90 95 100 105	
GGC GAG GCA GGG GCG TGG GGC GGC GAT CGC GAG CCG CCA GCC GCG GGC	627
Gly Glu Ala Gly Ala Trp Gly Gly Asp Arg Glu Pro Pro Ala Ala Gly	
110 115 120	
CCA CGG GCG CTG GGG CCG CCC GCC GAG GAG CCG CTG CTC GCC GCC AAC	675
Pro Arg Ala Leu Gly Pro Pro Ala Glu Glu Pro Leu Leu Ala Ala Asn	
125 130 135	
GGG ACC GTG CCC TCT TGG CCC ACC GCC CCG GTG CCC AGC GCC GGC GAG	723
Gly Thr Val Pro Ser Trp Pro Thr Ala Pro Val Pro Ser Ala Gly Glu	
140 145 150	
CCC GGG GAG GAG GCG CCC TAT CTG GTG AAG GTG CAC CAG GTG TGG GCG	771
Pro Gly Glu Glu Ala Pro Tyr Leu Val Lys Val His Gln Val Trp Ala	
Lys Val His Glu Val Trp Ala	
GGF II 01 & GGF II 11	
155 160 165	
GTG AAA GCC GGG GGC TTG AAG AAG GAC TCG CTG CTC ACC GTG CGC CTG	819
Val Lys Ala Gly Gly Leu Lys Lys Asp Ser Leu Leu Thr Val Arg Leu	
Ala Lys Asp Leu Leu Leu Xaa Val Leu	
GGF II 10	
170 175 180 185	
GGG ACC TGG GGC CAC CCC GCC TTC CCC TCC TGC GGG AGG CTC AAG GAG	867
Gly Thr Trp Gly His Pro Ala Phe Pro Ser Cys Gly Arg Leu Lys Glu	
Gly Ala Trp Gly Pro Pro Ala Phe Pro Val Xaa Tyr	
GGF II 03	
190 195 200	
GAC AGC AGG TAC ATC TTC TTC ATG GAG CCC GAC GCC AAC AGC ACC AGC	915
Asp Ser Arg Tyr Ile Phe Phe Met Glu Pro Asp Ala Asn Ser Thr Ser	
Tyr Ile Phe Phe Met Glu Pro Asp Ala Xaa Ser Ser Gly	
GGF II 02	
205 210 215	

Figure 24 C

CGC	GCG	CCG	GCC	GCC	TTC	CGA	GCC	TCT	TTC	CCC	CCT	CTG	GAG	ACG	GGC	963
Arg	Ala	Pro	Ala	Ala	Phe	Arg	Ala	Ser	Phe	Pro	Pro	Leu	Glu	Thr	Gly	
		220					225					230				
CGG	AAC	CTC	AAG	AAG	GAG	GTC	AGC	CGG	GTG	CTG	TGC	AAG	CGG	TGC	GCC	1011
Arg	Asn	Leu	Lys	Lys	Glu	Val	Ser	Arg	Val	Leu	Cys	Lys	Arg	Cys	Ala	
	235					240					245					
TTG	CCT	CCC	CAA	TTG	AAA	GAG	ATG	AAA	AGC	CAG	GAA	TCG	GCT	GCA	GGT	1059
Leu	Pro	Pro	Gln	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu	Ser	Ala	Ala	Gly	
	250				255					260					265	
TCC	AAA	CTA	GTC	CTT	CGG	TGT	GAA	ACC	AGT	TCT	GAA	TAC	TCC	TCT	CTC	1107
Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu	Tyr	Ser	Ser	Leu	
		Leu	Val	Leu	Arg											
		GGF	II	06												
			270					175						180		
AGA	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AAT	GAA	TTG	AAT	CGA	AAA	AAC	AAA	1155
Arg	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Asn	Glu	Leu	Asn	Arg	Lys	Asn	Lys	
			185					190					195			
CCA	CAA	AAT	ATC	AAG	ATA	CAA	AAA	AAG	CCA	GGG	AAG	TCA	GAA	CTT	CGC	1203
Pro	Gln	Asn	Ile	Lys	Ile	Gln	Lys	Lys	Pro	Gly	Lys	Ser	Glu	Leu	Arg	
		200					205					210				
ATT	AAC	AAA	GCA	TCA	CTG	GCT	GAT	TCT	GGA	GAG	TAT	ATG	TGC	AAA	GTG	1251
Ile	Asn	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Cys	Lys	Val	
		Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Xaa	Lys		
							GGF	II	12							
	215					220				225						
ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAT	ATC	ACC	ATC	GTG	1299
Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	Ile	Thr	Ile	Val	
	230				235					240					245	
GAA	TCA	AAC	GCT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	AGC	CAT	CTT	GTA	1347
Glu	Ser	Asn	Ala	Thr	Ser	Thr	Ser	Thr	Thr	Gly	Thr	Ser	His	Leu	Val	
				250					255					260		

Figure 24 D

AAA TGT GCG GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC	1395
Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys	
265 270 275	
TTC ATG GTG AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG TGC AAG TGC	1443
Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu Cys Lys Cys	
280 285 290	
CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA AAC TAC GTA ATG GCC AGC	1491
Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala Ser	
295 300 305	
TTC TAC AGT ACG TCC ACT CCC TTT CTG TCT CTG CCT GAA	1530
Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu	
400 405 410	
TAGGAGCATG CTCAGTTGGT GCTGCTTTCT TGTGCTGCA TCTCCCCTCA GATTCCACCT	1590
AGAGCTAGAT GTGTCTTACC AGATCTAATA TTGACTGCCT CTGCCTGTCG CATGAGAACA	1650
TTAACAAAAG CAATTGTATT ACTTCCTCTG TTCGCGACTA GTTGGCTCTG AGATACTAAT	1710
AGGTGTGTGA GGCTCCGGAT GTTTCTGGAA TTGATATTGA ATGATGTGAT ACAAATTGAT	1770
AGTCAATATC AAGCAGTGAA ATATGATAAT AAAGGCATTT CAAAGTCTCA CTTTATTGA	1830
TAAAATAAAA ATCATTCTAC TGAACAGTCC ATCTTCTTTA TACAATGACC ACATCCTGAA	1890
AAGGGTGTTG CTAAGCTGTA ACCGATATGC ACTTGAAAATG ATGGTAAGTT AATTTTGATT	1950
CAGAATGTGT TATTTGTCAC AAATAAACAT AATAAAAGGA AAAAAAAAAA AAA	2003





Figure 26

Met Arg Trp Arg Arg Ala Pro Arg Arg Ser Gly Arg Pro Gly Pro Arg  
 1 5 10 15  
 Ala Gln Arg Pro Gly Ser Ala Ala Arg Ser Ser Pro Pro Leu Pro Leu  
 20 25 30  
 Leu Pro Leu Leu Leu Leu Gly Thr Ala Ala Leu Ala Pro Gly Ala  
 35 40 45  
 Ala Ala Gly Asn Glu Ala Ala Pro Ala Gly Ala Ser Val Cys Tyr Ser  
 50 55 60  
 Ser Pro Pro Ser Val Gly Ser Val Gln Glu Leu Ala Gln Arg Ala Ala  
 65 70 75 80  
 Val Val Ile Glu Gly Lys Val His Pro Gln Arg Arg Gln Gln Gly Ala  
 85 90 95  
 Leu Asp Arg Lys Ala Ala Ala Ala Ala Gly Glu Ala Gly Ala Trp Gly  
 100 105 110  
 Gly Asp Arg Glu Pro Pro Ala Ala Gly Pro Arg Ala Leu Gly Pro Pro  
 115 120 125  
 Ala Glu Glu Pro Leu Leu Ala Ala Asn Gly Thr Val Pro Ser Trp Pro  
 130 135 140  
 Thr Ala Pro Val Pro Ser Ala Gly Glu Pro Gly Glu Glu Ala Pro Tyr  
 145 150 155 160  
 Leu Val Lys Val His Gln Val Trp Ala Val Lys Ala Gly Gly Leu Lys  
 165 170 175  
 Lys Asp Ser Leu Leu Thr Val Arg Leu Gly Thr Trp Gly His Pro Ala  
 180 185 190  
 Phe Pro Ser Cys Gly Arg Leu Lys Glu Asp Ser Arg Tyr Ile Phe Phe  
 195 200 205  
 Met Glu Pro Asp Ala Asn Ser Thr Ser Arg Ala Pro Ala Ala Phe Arg  
 210 215 220  
 Ala Ser Phe Pro Pro Leu Glu Thr Gly Arg Asn Leu Lys Lys Glu Val  
 225 230 235 240  
 Ser Arg Val Leu Cys Lys Arg Cys Ala Leu Pro Pro Gln Leu Lys Glu  
 245 250 255  
 Met Lys Ser Gln Glu Ser Ala Ala Gly Ser Lys Leu Val Leu Arg Cys  
 260 265 270  
 Glu Thr Ser Ser Glu Tyr Ser Ser Leu Arg Phe Lys Trp Phe Lys Asn  
 275 280 285  
 Gly Asn Glu Leu Asn Arg Lys Asn Lys Pro Gln Asn Ile Lys Ile Gln  
 290 295 300  
 Lys Lys Pro Gly Lys Ser Glu Leu Arg Ile Asn Lys Ala Ser Leu Ala  
 305 310 315 320  
 Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu Gly Asn Asp  
 325 330 335

**Figure 26 (cont.)**

Ser	Ala	Ser	Ala	Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Ala	Thr	Ser	Thr
			340					345					350		
Ser	Thr	Thr	Gly	Thr	Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys
			355				360					365			
Thr	Phe	Cys	Val	Asn	Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser
	370					375					380				
Asn	Pro	Ser	Arg	Tyr	Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp
385					390					395					400
Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala	Ser	Phe	Tyr	Ser	Thr	Ser	Thr	Pro
				405					410					415	
Phe	Leu	Ser	Leu	Pro	Glu	*									
			420												

Figure 27

TCTAA AAC TAC AGA GAC TGT ATT TTC ATG ATC ATC ATA GTT CTG TGA AAT ATA	53
Asn Tyr Arg Asp Cys Ile Phe Met Ile Ile Ile Val Leu Xaa Asn Ile	
1 5 10 15	
CTT AAA CCG CTT TGG TCC TGA TCT TGT AGG AAG TCA GAA CTT CGC ATT	101
Leu Lys Pro Leu Trp Ser Xaa Ser Cys Arg Lys Ser Glu Leu Arg Ile	
20 25 30	
AGC AAA GCG TCA CTG GCT GAT TCT GGA GAA TAT ATG TGC AAA GTG ATC	149
Ser Lys Ala Ser Leu Ala Asp Ser Gly Glu Ser Met Cys Lys Val Ile	
35 40 45	
AGC AAA CTA GGA AAT GAC AGT GCC TCT GCC AAC ATC ACC ATT GTG GAG	197
Ser Lys Leu Gly Asn Asp Ser Ala Ser Ala Asn Ile Arg Ile Val Glu	
50 55 60	
TCA AAC GGT AAG AGA TGC CTA CTG CGT GCT ATT TCT CAG TCT CTA AGA	245
Ser Asn Gly Lys Arg Cys Leu Leu Arg Ala Ile Ser Gln Ser Leu Arg	
65 70 75 80	
GGA GTG ATC AAG GTA TGT GGT CAC ACT TGA ATC ACG CAG GTG TGT GAA	293
Gly Val Ile Lys Val Cys Gly His Thr Xaa Ile Thr Gln Val Cys Glu	
85 90 95	
ATC TCA TTG TGA ACA AAT AAA AAT CAT GAA AGG AAA ACT CTA TGT TTG	341
Ile Ser Cys Xaa Thr Asn Lys Asn His Glu Arg Lys Thr Leu Cys Leu	
100 105 110	
AAA TAT CTT ATG GGT CCT CCT GTA AAG CTC TTC ACT CCA TAA GGT GAA	389
Lys Tyr Leu Met Gly Pro Pro Val Lys Leu Phe Thr Pro Xaa Gly Glu	
115 120 125	
ATA GAC CTG AAA TAT ATA TAG ATT ATT T	417
Ile Asp Leu Lys Tyr Ile Xaa Ile Ile	
130 135	

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/14974

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 48/00, 38/18

US CL : 424/93.21; 514/12, 44

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21; 514/12, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FASEB Journal, Volume 8, Numbers 4-5, issued 1994, Danilenko et al, "Neu Differentiation Factor (NDF) Accelerates Epidermal Migration and Differentiation in Excisional Wounds", page A535, see entire abstract.	36-37
A	BioEssays, Volume 15, Number 12, issued December 1993, Peles et al, "Neu and its Ligands: From an Oncogene to Neural Factors", pages 815-824, see entire document.	1-37
A, P	US, A, 5,367,060 (VANDLEN ET AL) 22 November 1994, see column 37, line 54 to column 39, line 45.	1-2, 7-32, 35-37

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 FEBRUARY 1996

Date of mailing of the international search report

14 MAR 1996

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/14974

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. P. BROCKES et al, "The Neuron as a Source of Mitogen: Its Influence on the Proliferation of Glial and Non-neural Cells," from "Development in the Nervous System", published 1980, Garrod and Feldman, (CA), pages 309-327, see entire document.	1-2, 7-32, 35-37
A, P	US, A, 5,399,346 (ANDERSON ET AL) 21 March 1995, see entire document.	3-6, 33-34
A	Proc. Natl. Acad. Sci. USA, Volume 89, issued April 1992, Yao et al, "Expression of Human Factor IX in Mice after Injection of Genetically Modified Myoblasts", pages 3357-3361, see entire document.	3-6, 33-34
A, P	SPECIAL NEWS REPORT, Volume 269, issued 25 August 1995, Marshall, "Gene Therapy's Growing Pains", pages 1050-1055, see entire document.	3-6, 33-34
A	US, A, 5,082,670 (GAGE ET AL) issued 21 January 1992, see entire document.	3-6, 33-34

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/14974

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS/USPAT, MEDLINE, BIOSIS, WORLD PATENT INDEX

Search terms: neuregulin#, heregulin#, glial growth factor#, acetylcholine receptor inducing activity, ARIA, neu differentiation factor#

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-2, 7-32, and 35-37, drawn to a method of affecting cellular communication in a vertebrate by administering a neuregulin protein.

Group II, claim(s) 3-6 and 33-34, drawn to a method of affecting cellular communication in a vertebrate by administering neuregulin-producing cells or neuregulin DNA (gene therapy).

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is the administration of neuregulin protein to vertebrates. The special technical feature of Group II is the administration of neuregulin DNA either directly (in vivo gene therapy) or after introduction of DNA into a cell (ex vivo gene therapy). The method of Group II does not require the administration of neuregulin protein and the method of Group I does not require the administration of neuregulin DNA. Furthermore, the method steps for administration of protein and DNA are different. Therefore, both the reagents used and the process steps of Groups I and II are distinct from one another and the special technical features of each method distinguish the two groups from one another. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.